PHD

Pharmacologial effects of the epoxyeicosatrienoic acids

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PHARMACOLOGICAL EFFECTS OF THE EPOXYEICOSATRIENOIC ACIDS

submitted by M.F. Clegg

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TO MY WIFE AMANDA

SUMMARY

The epoxyeicosatrienoic acids (EETs) are a group of four regio-isomeric cytochrome P_{450} metabolites of arachidonic acid. Since their first isolation and identification in rabbit hepatic microsomal incubation medium in 1982, investigations into their biological functions have been limited.

Following the successful synthesis of the EETs, separation by TLC and identification by GC-MS, all four isomers were tested for biological activity in rat thoracic aortic strips, guinea-pig tracheal rings and rat washed platelets.

All four EETs were found to relax phenylephrine pre-contracted aortic strips in a dose-dependent manner. 5(6)-EET was observed to be significantly more potent than the other three isomers especially in aortic strips with an intact endothelial lining. Removal of the endothelium was found to reduce the potency of 5(6)-EET whilst having no effect on the relaxations induced by the other EETs. Inhibition of cyclo-oxygenase activity with indomethacin also inhibited 5(6)-EET-induced relaxations, whereas NDGA, a lipoxygenase inhibitor, had no effect on any of the EET-induced responses. Inhibition of cGMP-phosphodiesterase by M&B 22,948 increased the potency of 8(9)-, 11(12)- and 14(15)-EET.

The EET-induced relaxatory responses induced in guinea-pig tracheal rings were similar to those in rat

aortic strips. Again the 5(6)-isomer was more potent than the other EETs. Indomethacin attenuated the potency of 5(6)-EET whilst augmenting the maximal response, however, NDGA did not effect EET-induced relaxant responses. M&B 22,948, in an analogous manner to that observed in the aorta, increased the potency of 8(9)- 11(12)- and 14(15)-EET in guinea-pig tracheal rings.

Platelet aggregation induced by both ADP and thrombin was inhibited by the EETs, 5(6)-EET being the most potent of the EETs. However, in platelets, neither indomethacin or NDGA had any significant effect on the inhibition induced by any of the EETs. Papaverine was observed to augment the inhibition induced by all four EETs, whilst M&B 22,948 did not alter the anti-aggregatory responses.

CONTENTS

The numbering of the tables in the appendix 2 are co-ordinated in order to ease cross-reference (i.e. data to Fig.3.21 can be found in table A3.21).

CHAPTER 1: INTRODUCTION

The metabolism of arachidonic acid (AA), a 20-carbon polyunsaturated essential fatty acid (EFA), to prostaglandins (PGs), thromboxanes (TXs) and leukotrienes (LTs), and the biological importance of these compounds has been the centre of intense pharmacological investigation.

The importance of the EFAs for normal body function was recognised in the 1930's by Burr & Burr (1930) whilst working on vitamin E and ovulation in rats. Around the same time, the biological actions of PGs were described by U.S. von Euler and by M.W. Goldblatt (Bergstrom & Sjovall, 1957), however their structures were undetermined. It was nearly thirty years later that PGE_1 and $PGF_{1\alpha}$ were isolated (Bergstrom & Sjovall, 1957). The link between EFAs and PGs was soon revealed and the synthesis of PGE_2 achieved by Bergstrom et al. (1964) and Van Dorp et al. (1964). Thromboxane A_2 (TXA_2), a potent aggregator of platelets and its breakdown product thromboxane B_2 (TXB_2) were isolated ten years later (Hamberg et al., 1975). Shortly afterwards Moncada and colleagues (1976) identified prostacyclin (PGI_2), a labile two cyclo-pentane ring structure with anti-aggregatory activity. A second pathway for AA metabolism, via 5-lipoxygenase producing the leukotrienes (LTs) was later described (Bray, 1983; Piper, 1983; Taylor & Morris, 1983).

In the early 80's, a third pathway of AA metabolism via cytochrome P_{450} mono-oxygenase was described (Oliw et al., 1981). The products of this pathway include a series of epoxides together with their respective diols and some w- and (w-1)-hydroxylated compounds. During the mid 1980's reports of biological activity of the epoxides started to appear.

The purpose of this project is therefore to consolidate some previous investigations into epoxy-eicosatrienoic acid-induced biological effects and to extend investigations to other tissues. Furthermore the mechanism of action of these products will be examined with the aim of setting foundations for the clinical manipulation of the mono-oxygenase pathway.

The starting point for the biological synthesis of the epoxyeicosatrienoates (EETs), as for other eicosanoids is arachidonic acid. The opening sections of this introduction will therefore endeavour to familiarise the reader with the biological processes involved in the storage, release and subsequent metabolism of AA. Subsequently the biological distribution and physiological role of the EETs will be discussed.

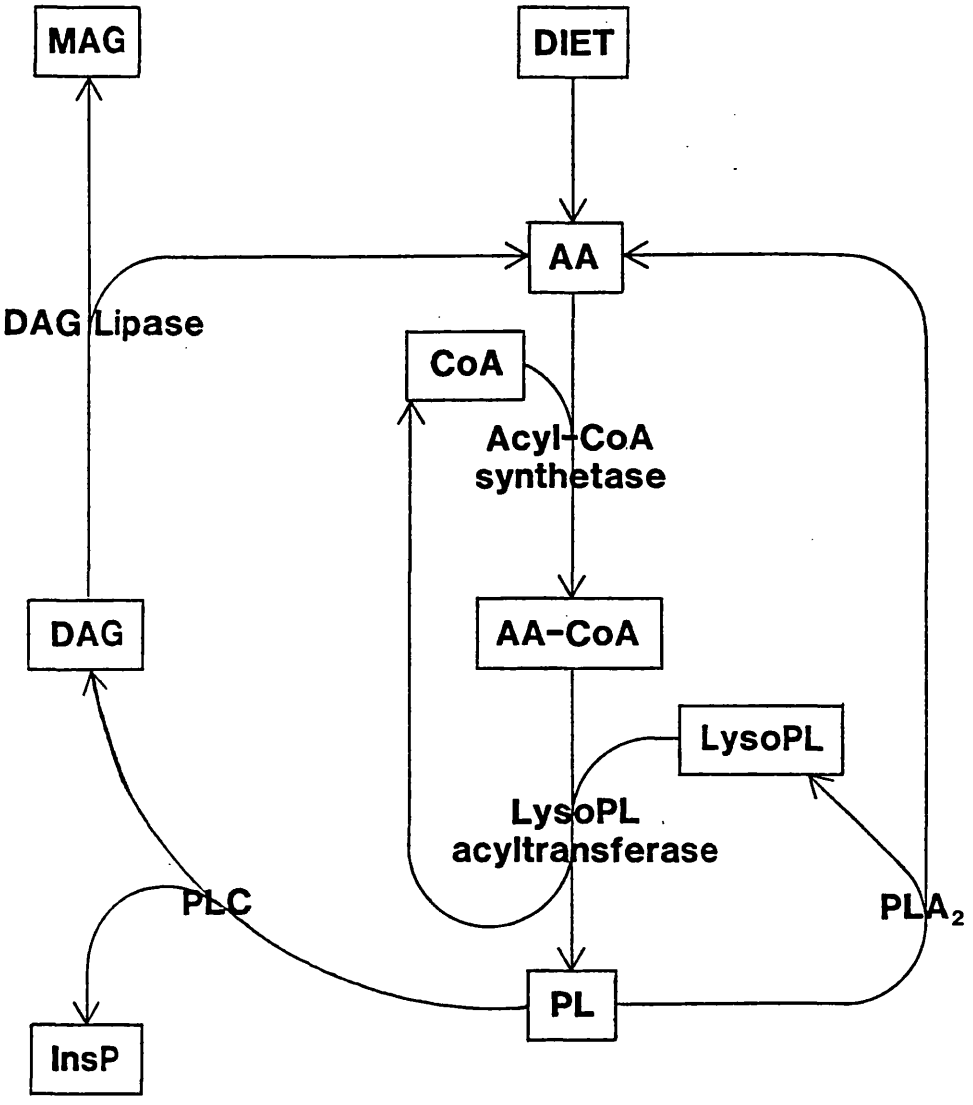
1.1: Arachidonic Acid Storage and Release

Under normal physiological conditions AA is found predominantly bound to the membrane phospholipids phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine and phosphatidylinositol. The enzyme responsible for incorporating AA into the phospholipids is an acyltransferase. The free acid is firstly bound to coenzyme-A (Co-A) by the action of acyl-CoA synthetase. Acyl-CoA synthetase does not appear to have any selectivity as to which fatty acids it utilises (Normann et al., 1981). AA can then be transferred to a lysophospholipid by lyso-phospholipid acyltransferase which has a high affinity selectively for AA-CoA (Jezky & Lands, 1968; Hasegawa-Sakai & Ohno, 1980), which increases further when lysophosphatidylinositol is the receptor (Baker & Thompson, 1973; Holub, 1976) (Fig. 1.1). The phosphatidylinositols are therefore a major source of arachidonic acid. AA-CoA specificity may be due to a separate enzyme (Hasegawa-Sakai & Ohno, 1980) and could explain the disproportionately large quantities of AA esterified to the inositol phosphates (Irvine, 1982).

Under resting conditions there is a small release of AA setting up a dynamic equilibrium, with the uptake mechanism, which controls the actual amount of free AA in cells at any given time (Irvine, 1982). The free levels of AA have been shown to be of paramount importance in controlling the formation of any AA-metabolites (Flower &

FIGURE 1.1

Incorporation and hydrolytic cleavage of AA from membrane phospholipid stores. Abbreviations: AA, arachidonic acid; CoA, coenzyme-A; PL, phospholipid; PLA_2, phospholipase A_2; PLC, phospholipase C; InsP, inositol trisphosphate; DAG, diacylglycerol; MAG, monoacylglyecrol.


MAG
DIET
AA
DAG Lipase
CoA
Acyl-CoA
synthetase
DAG
AA-CoA
LysoPL
LysoPL
acyltransferase
PLC
PLA2
PL
InsP

Blackwell, 1976; Irvine, 1982). On stimulation of the cell membrane the balance is shifted in favour of AA release (Broekman et al., 1981; Rittenhouse-Simmons, 1981; Billah & Lapetina, 1982a; Emilsson & Sundler, 1986), however the phospholipase involved is still controversial. Possible pathways of AA release from the phospholipid stores include:

i) phospholipase A_1 (PLA_1)/lyso phospholipase (PLB),

ii) phospholipase A_2 (PLA_2),

iii) phospholipase C (PLC)/diacylglycerol (DAG) lipase,

iv) PLC/DAG kinase/PLA_2.

(Fig. 1.2).

After cell activation, the release of AA is limited by an increase in acyltransferase activity (Schrey & Rubin, 1979).

1.1.1: PLA_1/PLB

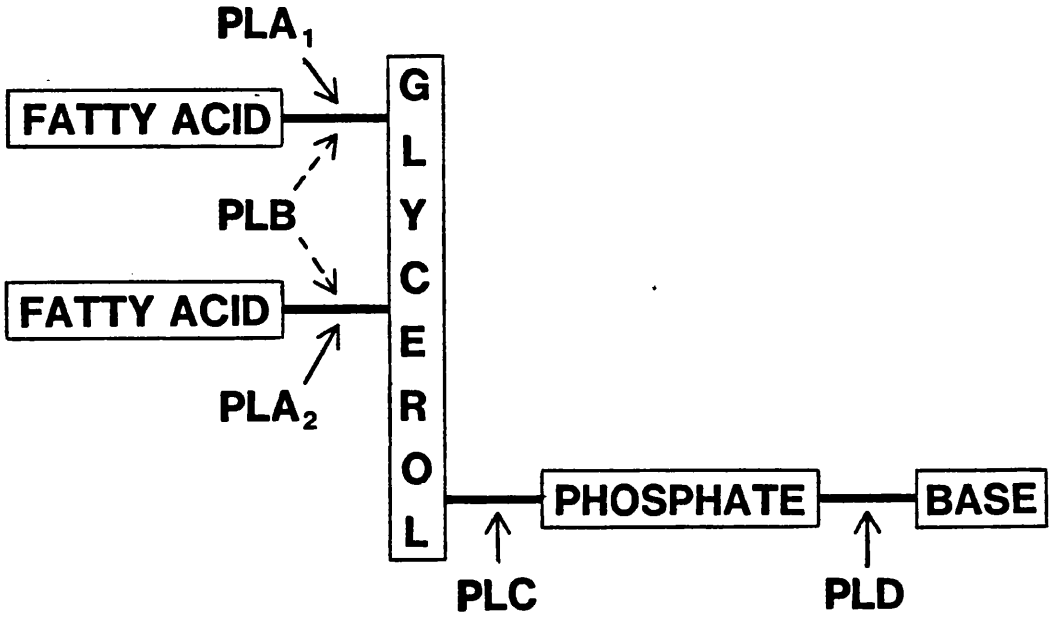
The Ca^{2+}-independent, lysosomal enzyme, PLA_1 was found to have a pH optimum of 4.8, whilst PLB was optimum at pH 8.5. At the low pH conditions required for PLA_1, PLB was virtually inactive, in addition to which the activity of PLA_1 was still only one quarter that of PLA_2. As a consequence, little time has been spent investigating this enzyme system as a possible release mechanism for AA.

1.1.2: PLA_2

As the majority of arachidonate is stored in the 2-acyl position of phospholipids the involvement of PLA_2 in its release was suggested by Lands & Samuelsson

FIGURE 1.2

Points of phospholipase attack on membrane lipids. PLB hydrolysis of lyso-phospholipids follows PLA_1 or PLA_2 hyrolysis of parent phospholipid. Abbreviations: PLA_1, phospholipase A_1; PLA_2, phospholipase A_2; PLB, lyso phospholipase; PLC, phospholipase C; PLD, phospholipase D.


PLA1
FATTY ACID
PLB
FATTY ACID
PLA2
GLYCEROL
PHOSPHATE
BASE
PLC
PLD

(1968). When phosphatidylcholine and phosphatidyl-ethanolamine are substrates it is now widely accepted that AA is liberated by the action of phospholipase A_2 (PLA_2). There is also evidence that PLA_2 is, at least in part, responsible for the liberation of AA from phosphatidylinositol (Flower & Blackwell, 1976; Blackwell et al., 1977; Hong & Deykin, 1981; Billah & Lapetina, 1982a).

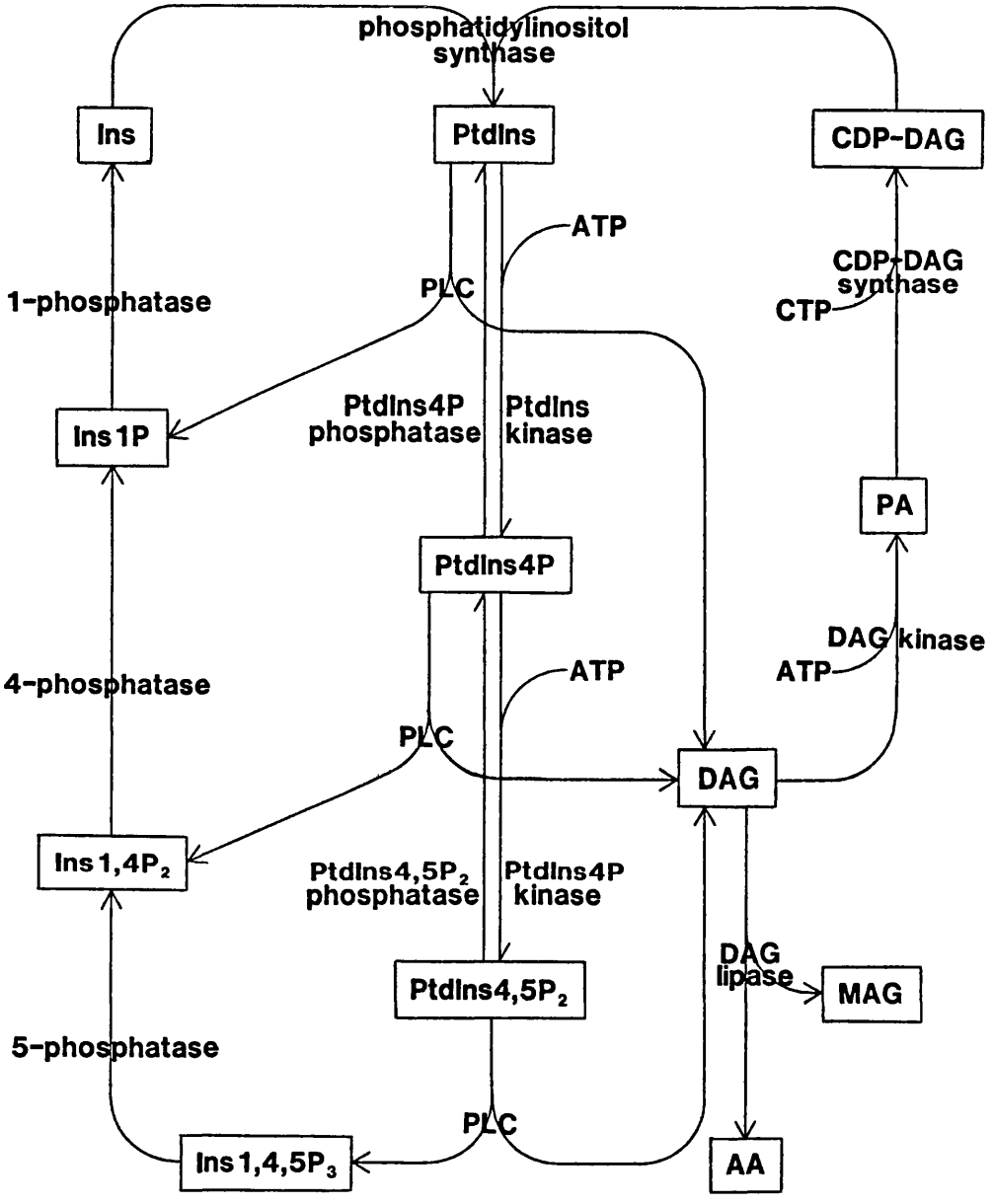
1.1.3: PLC/ DAG lipase

A calcium-independent phosphodiesterase, PLC, attacks phosphatidylinositol 4,5-bisphosphate (PtdIns4,5P_2) at the sn-3 position to release 1,2-DAG and inositol 1,4,5-trisphosphate ($InsP_3$) (Berridge, 1983). The breakdown of PtdIns4,5P_2 precedes the disappearance of phosphatidylinositol (PtdIns) and phosphatidyl 4-monophosphate (PtdIns4P) (Rebecchi & Gershengorn, 1983). 1,2-DAG can then be metabolised by 1,2-DAG lipase to 2-monoacylglycerol (2-MAG), the 2-MAG liberating AA and glycerol due to the action of 2-MAG lipase (Bell et al., 1979; Prescott & Majerus, 1983). (Fig. 1.3).

The AA liberation from phosphatidylinositol alone is not enough to account for all the AA released following cell stimulation, however it does precede and possibly triggers AA release by other phospholipases from other phospholipids (e.g. phosphatidycholine).

FIGURE 1.3

Inositol phosphate cycle. Abbreviations: PtdIns, phosphatidylinositol; PtdIns4P, phosphatidylinositol 4-monophosphate; PtdIns4,5P_2, phosphatidylinositol 4,5-bisphosphate; ATP, adenosine triphosphate; PLC, phospholipase C; Ins1,4P_2, inositol 1,4-bisphosphate; Ins1P, inositol phosphate; Ins, inositol; DAG, diacylglycerol; PA, phosphatidic acid; CDP, cytosine diphosphate; MAG, monoacylglycerol; AA, arachidonic acid.


phosphatidylinositol synthase
Ins
PtdIns
CDP-DAG
ATP
CDP-DAG synthase
CTP
1-phosphatase
PLC
Ins1P
PtdIns4P phosphatase
PtdIns kinase
PA
PtdIns4P
DAG kinase
ATP
4-phosphatase
ATP
PLC
DAG
Ins1,4P2
PtdIns4,5P2 phosphatase
PtdIns4P kinase
DAG lipase
MAG
PtdIns4,5P2
5-phosphatase
PLC
Ins1,4,5P3
AA

1.1.4: PLC/ DAG kinase & PLA_2

As above, the activity of PLC would liberate DAG. DAG could alternatively be phosphorylated by DAG kinase to phospatidic acid. Appearance of phosphatidic acid before the liberation of AA has been reported (Lapetina & Cuatrecasas, 1979). Liberated phosphatidic acid and $InsP_3$ may open calcium gates allowing the activation of PLA_2 which would release AA from phosphatidic acid or any of the phospholipids (Lapetina et al., 1981).

1.2: Arachidonic acid metabolism

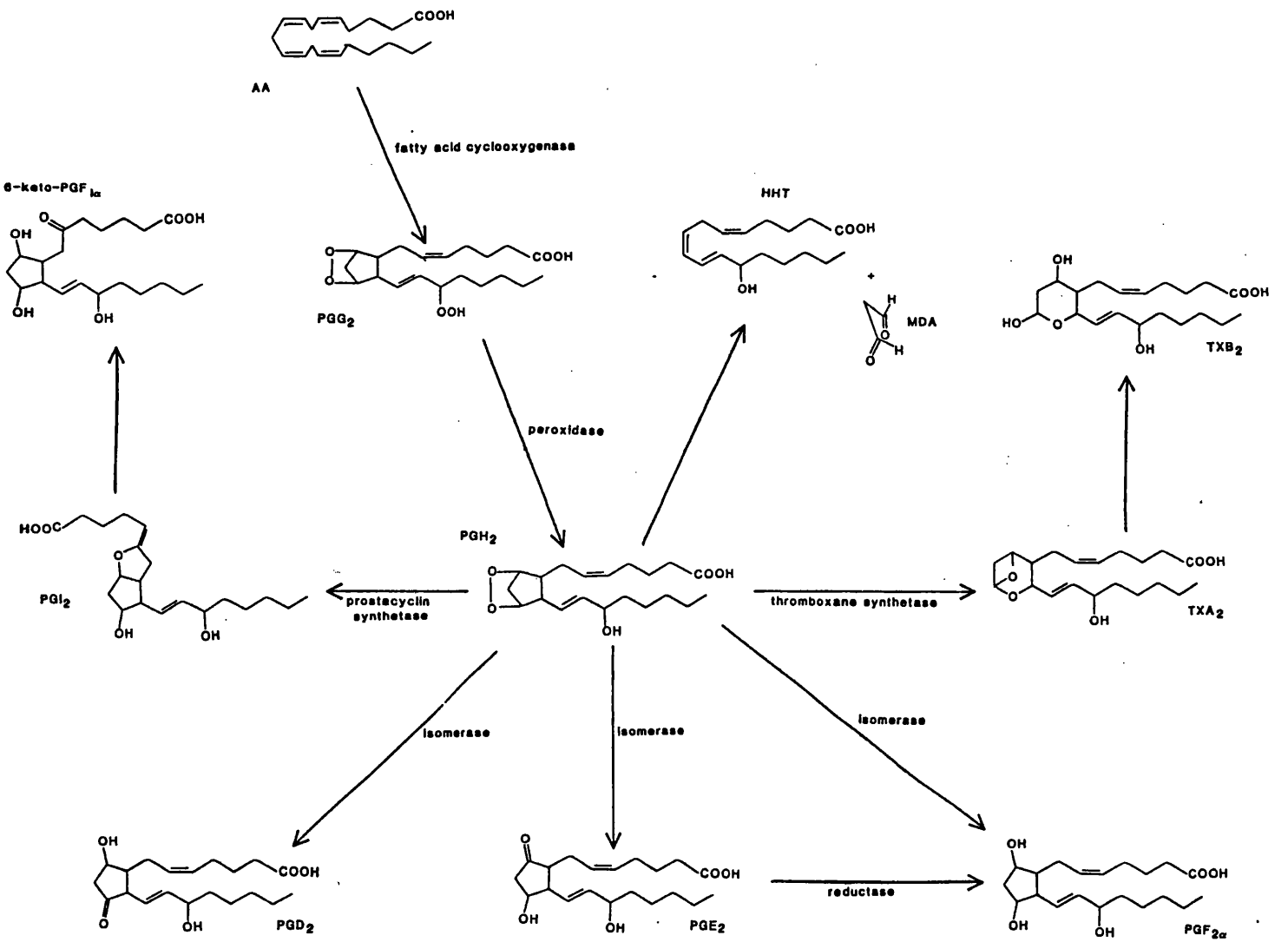
Once cellular levels of free AA have increased, free AA is rapidly converted by several different enzyme systems to many physiologically active metabolites. The more commonly known and widely investigated AA metabolites are the eicosanoids. Indeed the rate determining step in the production of the eicosanoids is the liberation of free AA (Flower & Blackwell, 1976). The eicosanoids include the prostaglandins (PGs), prostacyclin (PGI_2), thromboxanes (TXAs) and leukotrienes (LTs) (Hamberg & Samuelsson, 1974; Hamberg et al., 1975; Moncada et al., 1976), alternatively AA can be metabolised by the cytochrome P_{450} pathway (see later).

1.2.1: Cyclo-oxygenase Pathway

The first step in the 'cyclic pathway' is the oxidation of AA under the control of fatty acid cyclo-oxygenase (Lands, 1979). The resulting prostaglandin endoperoxide, PGG_2, is then reduced by peroxidase activity to a second endoperoxide, PGH_2 (Hamberg et al., 1974; Nugteren & Hazelhof, 1973). Further conversion of the endoperoxides can proceed either non-enzymatically or enzymatically as a result of isomerase activity. The resulting products, the 'primary prostaglandins', consist of PGD_2, PGE_2 and $PGF_{2\alpha}$. The conversion of PGE_2 to $PGF_{2\alpha}$ by 9-oxoprostaglandin reductase and of $PGF_{2\alpha}$ to PGE_2 by 9-hydroxyprostaglandin dehydrogenase has been demonstrated (Lands, 1979;

Pace-Asciak, 1975), but the biological relevance is unknown. Other prostaglandins, namely PGA_2 and B_2 are breakdown products of PGE_2 formed during chemical isolation, PGC_2 being enzymatically derived from PGA_2. Two other important products of the endoperoxides are prostacyclin (PGI_2) and thromboxane A_2 (TBA_2). Their formation is governed by prostacyclin synthetase and thromboxane synthetase respectively (Hamberg & Samuelsson, 1974; Hamberg et al., 1975) . Both compounds are unstable, PGI_2 hydrolyses non-enzymatically to 6-oxo-$PGF_{1\alpha}$ whilst TXA_2 breaks down non-enzymatically to TXB_2. The relative proportions of PGs produced varies between tissues, furthermore the resultant mixture may even vary within a tissue depending upon the level of available substrate. One further cyclo-oxygenase product is 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT), a 17-carbon hydroxy fatty acid, together with malondialdehyde (MDA). HHT and MDA are formed non-enzymatically from PGH_2. (Fig. 1.4).

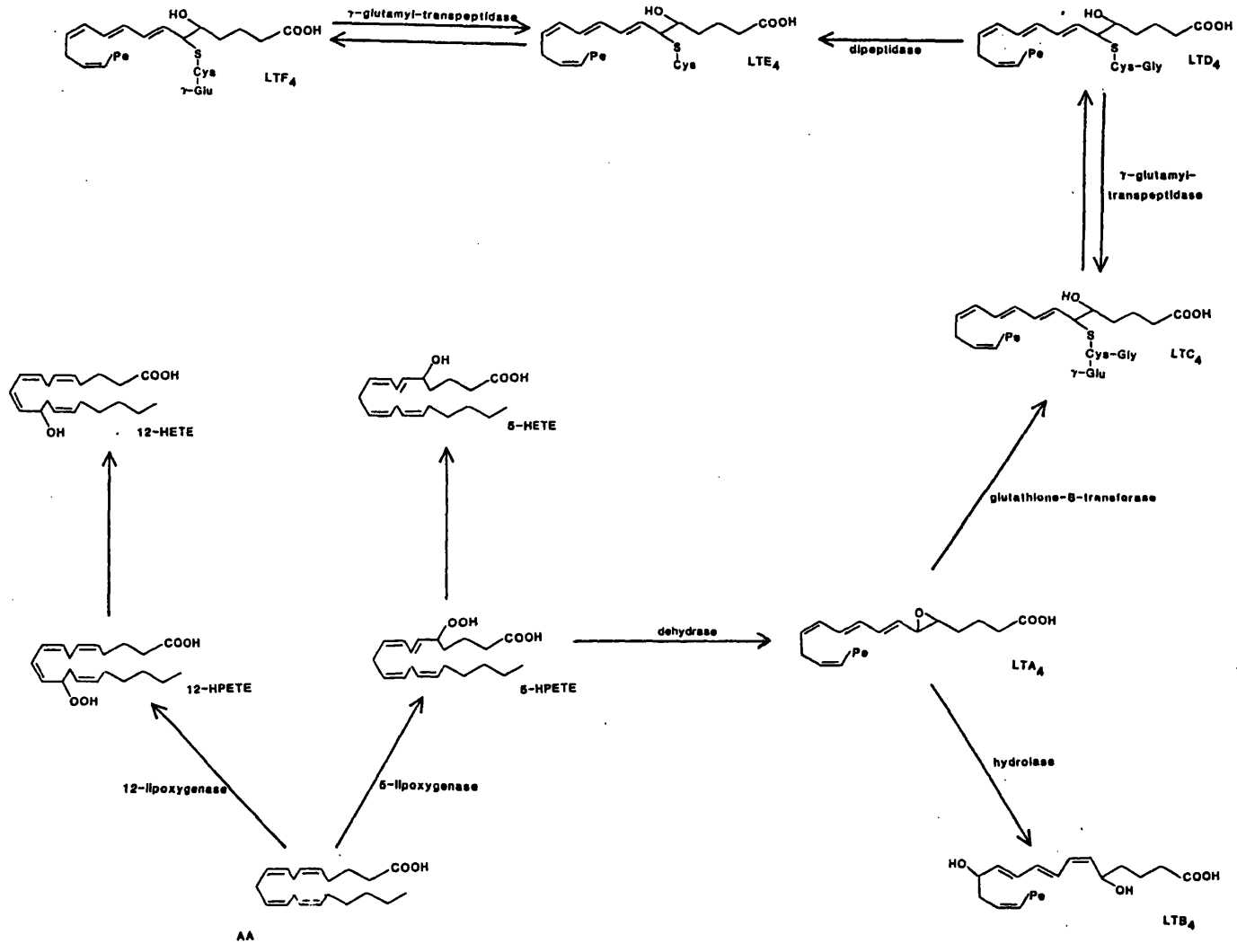
FIGURE 1.4
Cyclo-oxygenase pathway. Abbreviations: AA, arachidonic acid; PG, prostaglandin; TX, thromboxane; HHT, heptadecatrienoic acid; MDA, malondialdehyde.


AA
fatty acid cyclooxygenase
6-keto-PGF1α
PGG2
HHT
MDA
TXB2
peroxidase
PGH2
PGI2
prostacyclin synthetase
thromboxane synthetase
TXA2
isomerase
isomerase
isomerase
PGD2
PGE2
reductase
PGF2α

1.2.2: Lipoxygenase Pathway

The 'linear pathway' is initiated by 12-lipoxygenase or 5-lipoxygenase which may metabolise, amongst other fatty acids, AA. The essential structural requirement for the hydroperoxidation is a penta-1,4-*cis*-diene system (Taylor & Morris, 1983). 12-lipoxygenase converts AA to 12-hydroperoxyeicosa-5,8,10,14-tetraenoic acid (12-HPETE) (Nugteren, 1975), the reduction of which gives 12-hydroxyeicosa-5,8,10,14-tetraenoic acid (12-HETE). 5-lipoxygenase produces 5-hydroperoxyeicosa-6,8,11,14-tetraenoic acid (5-HPETE) (Borgeat et al., 1976), the reduction of which is catalysed by glutathione peroxidase (Bryant & Bailey, 1980) resulting in the hydroxy acid, 5-hydroxyeicosa-6,8,11,14-tetraenoic acid (5-HETE). Removal of water from 5-HPETE by dehydrase results in the formation of a 5,6-epoxide, leukotriene A_4 (LTA_4). Rehydrolysis of LTA_4 by hydrolase produces 5,12-dihydroxyeicosatetraenoic acid (leukotriene B_4, LTB_4). Alternatively LTA_4 may be conjugated with glutathione to form leukotriene C_4 (LTC_4), the enzyme responsible being glutathione-S-transferase. Subsequent cleavage of glutamic acid followed by glycine from LTC_4 leaves leukotriene D_4 (LTD_4) and leukotriene E_4 (LTE_4) respectively. Finally, re-incorporation of y-glutamic acid into LTE_4 produces leukotriene F_4 (LTF_4). (Fig. 1.5).

FIGURE 1.5
Lipoxygenase pathway. Abbreviations: AA, arachidonic acid; HPETE, hydroperoxyeicosatetraenoic acid; HETE, hydroxyeicosatetraenoic acid; LT, leukatriene.


γ-glutamyl-transpeptidase
dipeptidase
γ-glutamyl-transpeptidase
glutathione-S-transferase
dehydrase
12-lipoxygenase
5-lipoxygenase
hydrolase
LTF4
LTE4
LTD4
LTC4
LTA4
LTB4
12-HETE
5-HETE
12-HPETE
5-HPETE
AA
Pe
Cys
γ-Glu
Cys-Gly
COOH
OH
OOH
HO

1.3: Cytochrome P_{450}.

In addition to its metabolism by cyclo-oxygenase and lipoxygenase enzymes, unesterified AA may undergo oxidation by the cytochrome P_{450} mono-oxygenase system. Before describing the AA-products so formed, it is necessary to briefly describe the P_{450} mono-oxygenase pathway.

Cytochrome P_{450} is a primary component of the oxidative enzyme system found in the microsomal fraction of cells. Responsible for the metabolism of both endogenous and exogenous compounds it is located predominantly in the hepatocytes. However cytochrome P_{450} activity has also been described in lung, kidney, small intestine, placenta, skin and vascular tissue (Bond et al., 1979, 1980; Ullrich et al., 1981; Dees et al., 1982). Although cytochrome P_{450} has many isozymic forms, they all have molecular weights of 48-55 Kdaltons and possess a haem group ionically bound to an amino acid residue in the protein. Carbon monoxide (CO) binds avidly with cytochrome P_{450} causing enzyme inhibition, the resultant complex absorbing light of wavelength 450nm (hence cytochrome P_{450}). Cyanide on the other hand has no effect on microsomal oxidations, differentiating them from mitochondrial transformations. When the enzyme is isolated from the membrane (using surfactants), its UV absorption maximum drops to 420nm, the spectral properties altering again on association with a substrate

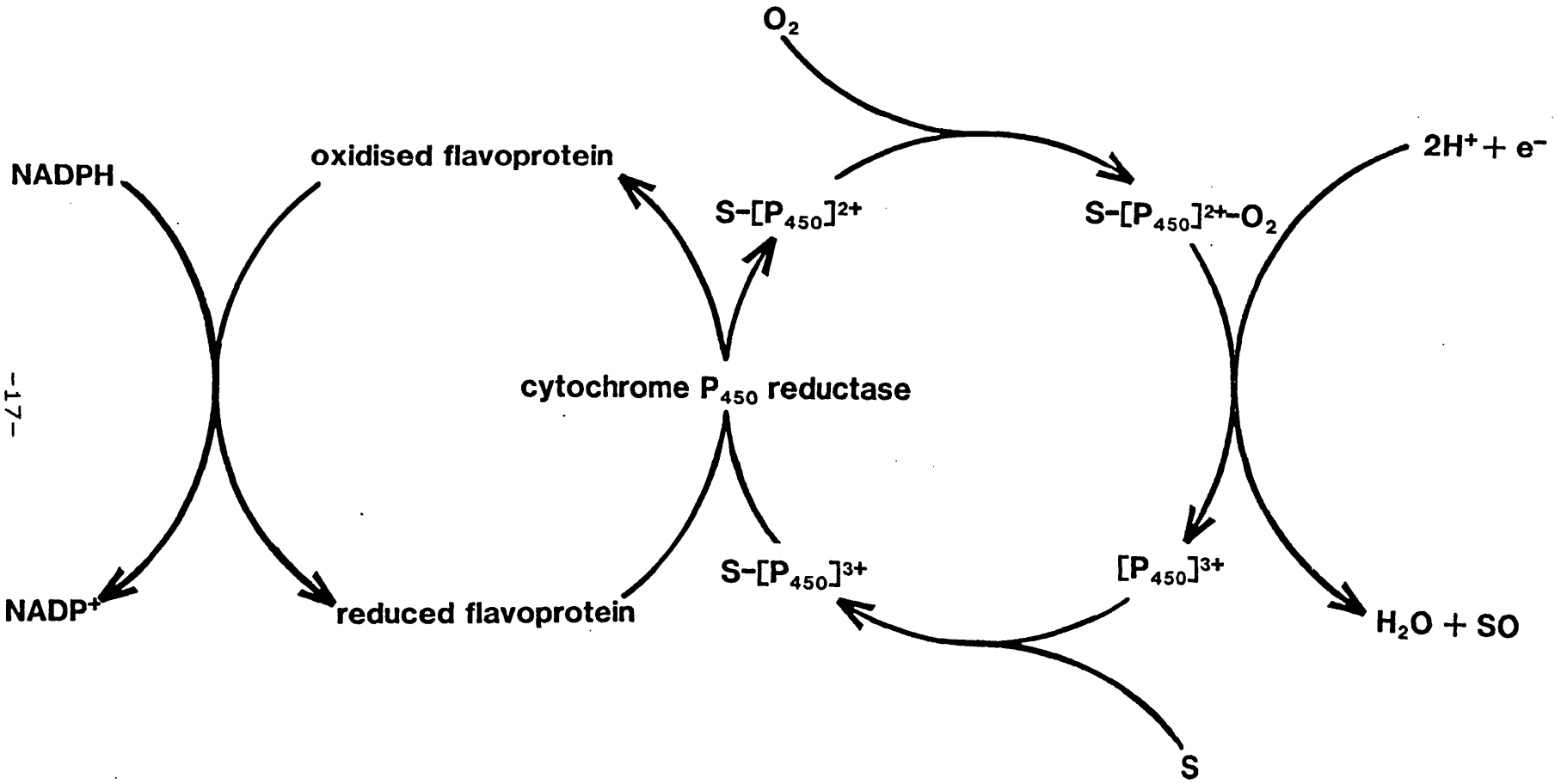
suggesting a conformational change on binding. Despite the large range of substrates (e.g. benzene derivatives and polycyclic aromatic hydrocarbons, alkyl and alkene chains, amines) metabolised by cytochrome P_{450}, they are all lipophilic, a physical property of paramount importance if they are to cross the cell membrane.

The oxidation process can be split into five stages starting with the binding of the substrate to oxidised P_{450}. The enzyme-substrate complex is reduced (the single electron donated by NADPH, via a flavoprotein) then bound with molecular oxygen (O_2). A second reduction takes place when two hydrogen atoms together with an electron are inserted resulting in the subsequent release of oxidised substrate together with water. The oxidised enzyme is also regenerated (Fig. 1.6).

Recently the Committee of Standardized Nomenclature of the P_{450} Genes was set up to oversee the naming of P_{450} isozymes. The agreed system involves the division of the P_{450} gene superfamily into families, subfamilies and genes. Gene families were designated Roman numerals, subfamilies designated capital letters and the individual genes were labelled using Arabic numerals e.g. rat P_{450}PB1 is P_{450}IIC6 (Nebert et al., 1987).

FIGURE 1.6

Cytochrome P_{450} pathway. Abbreviations: NADPH, reduced nicotinamide adenine dinucleotide phosphate; $NADP^+$, nicotinamide adenine dinucleotide phosphet; S, substrate; P_{450}, cytochrome P_{450} monooxygenase.


O2
NADPH
oxidised flavoprotein
S-[P450]2+
S-[P450]2+-O2
2H+ + e-
cytochrome P450 reductase
NADP+
reduced flavoprotein
S-[P450]3+
[P450]3+
H2O + SO
S

1.4: Cytochrome P_{450} Synthesis of the Epoxyarachidonates

The first work identifying NADPH-dependent cytochrome P_{450} metabolites of AA was described by Oliw et al. (1981). Both w- and (w-1)-hydroxylation products were identified following the incubation of AA with NADPH-fortified renal cortical microsomes. Previous work with medium chain (C8-C18) fatty acids had revealed similar compounds (Navarro et al., 1978). An unexpected result was the appearance of 11,12- and 14,15-dihydroxy-eicosatrienoic acids (DHETs). Oliw et al. (1981) proposed that these 1,2-diols were formed via their respective epoxides. Inhibition by carbon monoxide of the production of the diols confirmed their synthesis by cytochrome P_{450} mono-oxygenase activity.

Further work with hepatic microsomes showed that the two 1,2-diols could be further metabolised to their w- and (w-1)-hydroxylated products (Oliw and Oates, 1981). Furthermore the (w-1)-hydroxylated products could be converted to epoxyderivatives. In the same work Oliw and Oates confirmed that the two 1,2-diols were formed via their respective epoxyeicosatrienoic acids.

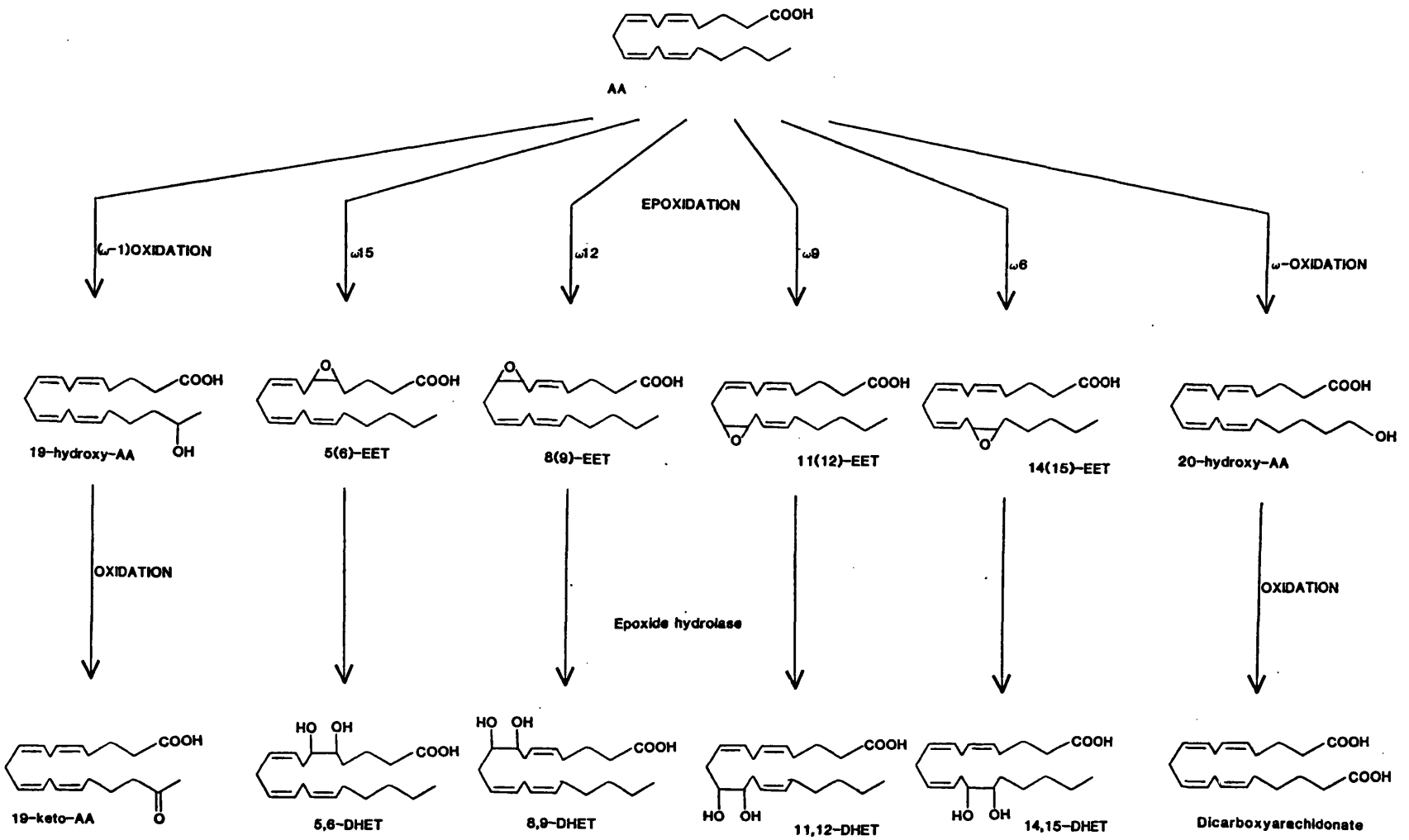
The 5(6)- and 8(9)-EETs were later isolated from hepatic microsomal incubations of AA in the presence of NADPH (Oliw et al., 1982). In order to isolate the four EETs rather than their corresponding 1,2-diols from microsomal incubations, it was necessary to use 1,2-epoxy-3,3,3-trichloropropane to inhibit epoxide

hydrolase, or to use purified cytochrome P_{450}, which proved more effective. It was speculated that the EETs were hydrolysed to the 1,2-diols by the action of microsomal epoxide hydrolase (Oliw et al., 1982). Ferrous iron or ADP were not important in the synthesis (Oliw et al., 1982). The 'epoxygenase' pathway is summarised in Fig. 1.7.

Work by Chacos et al., 1983 disputed the role of microsomal epoxide hydrolase in the hydration of epoxyeicosatrienoic acids as proposed by Oliw et al. (1981). The newly discovered cytosolic epoxide hydrolase was found to be far more active than its microsomal counterpart at hydrating the 8(9)-, 11(12)- and 14(15)-EETs, however the 5(6)-EET had a much lower reactivity to the cytosolic form. The poor metabolism of 5(6)-EET by epoxide hydrolase would therefore leave this cytochrome P_{450} product available to act on other physiological systems (see later). It was noted however that a tightly bound microsomal epoxide hydolase was present which possessed many of the cytosolic enzyme's properties and could be responsible for the misinterpretation of results obtained by Oliw et al. (1982).

Synthesis of the EETs by oxygen radical attack of AA was shown not to be involved by Capdevila et al. (1981a). The addition of mannitol, catalase or superoxide dismutase (SOD) had no effect on the turnover of AA in microsomal incubations. If oxygen reduction products were

FIGURE 1.7
Epoxygenase pathway. Abbreviations: AA, arachidonic acid; EET, epoxyeicosatrienoic acid; DHET, dihydroxy-eicosatrienoic acid.


COOH
AA
EPOXIDATION
(ω-1)OXIDATION
ω15
ω12
ω9
ω6
ω-OXIDATION
19-hydroxy-AA
OH
5(6)-EET
8(9)-EET
11(12)-EET
14(15)-EET
20-hydroxy-AA
OXIDATION
Epoxide hydrolase
OXIDATION
19-keto-AA
O
HO OH
5,6-DHET
8,9-DHET
11,12-DHET
14,15-DHET
Dicarboxyarachidonate

involved then a reduction in AA turnover would have been observed. Furthermore benzphetamine, a P_{450} substrate known to uncouple cytochrome P_{450} whilst increasing the production of oxygenated products by hydrogen peroxide, was found to reduce the turnover of AA, consolidating the view that oxygen radical attack was not involved.

The rate of AA metabolism by both kidney and liver microsomal P_{450} was increased 2 to 3 fold by the presence of cytochrome b_5 (Capdevila et al., 1981b). NADPH-fortified cytochrome b_5 alone was unable to metabolise AA indicating a separate metabolic path for b_5 was not responsible for the increase. The liver microsomal enzyme appeared to be more efficient at producing the EETs than did the kidney microsomal enzyme.

1.5: Metabolism of Cytochrome P_{450} Metabolites of Arachidonic Acid

As 5(6)-EET possesses three *cis* double bonds (a structural requirement of substrates for fatty acid cyclo-oxygenase) Oliw investigated the further epoxide metabolism by cyclo-oxygenase. Initial work utilising ram seminal vesicles (RSVs) indeed found 5(6)-EET to be converted to several polar products, the two major metabolites being identified as 5-hydroxy-prostaglandin $I_{1\alpha}$ and 5-hydroxy-prostaglandin I_{1b} (Oliw, 1984a). Production of these two metabolites was postulated to be via 5(6)-epoxyprostaglandin endoperoxides, 5(6)-epoxy-prostaglandin $F_{1\alpha}$ or both. Isolation of the two epoxyprostaglandin endoperoxides was later achieved following further arachidonic acid incubations with RSVs (Oliw, 1984d). 5(6)-epoxy-$PGF_{1\alpha}$ was also isolated from RSV incubations with 5(6)-EET, which was increased several fold in the presence of dithiothreitol (Oliw & Benthin, 1985), a compound known to increase the production of PGF compounds. In the presence of glutathione the major fatty acid cyclo-oxygenase metabolites of 5(6)-EET were found to be 5(6)-epoxyprostaglandin E_1, 5,6-dihydroxyprostaglandin E_1 and its d5-lactone (Oliw, 1984b). The enzyme endoperoxidase E isomerase with glutathione as a cofactor was probably responsible for this conversion as non-enzymatic breakdown of the epoxyprostaglandin

endoperoxides only occurs in the absence of cofactor (Oliw, 1984b). The epoxyendoperoxides were also found to be transformed into 5(6)-epoxy-8,11-heptadecadienoic acid (5(6)-epoxy-HHD) in small amounts (Oliw, 1984d).

5,6-DHET, the hydration product of 5(6)EET, has a metabolic fate parallel to 5(6)-EET. Fatty acid cyclo-oxygenase converted the 1,2-diol to 5,6-dihydroxyprostaglandin G_1 and 5,6-dihydroxy-prostaglandin H_1 (Oliw, 1984c). The 5,6-dihydroxy-prostaglandin endoperoxides may either undergo further enzymatic conversion to 5,6-dihydroxyprostaglandin E_1 by prostaglandin E isomerase or breakdown non-enzymatically to 5,6-dihydroxyprostaglandin $F_{1\alpha}$ (Oliw, 1984c). However the conversion to prostaglandin E compounds was found to be slower for 5,6-DHET than for 5(6)-EET or AA. Possible conversion of 5,6-DHET by either thromboxane or prostacyclin synthetase was not investigated.

In addition there is evidence that at least one of the isomers, the 8(9)-EET, is a substrate for soyabean 15-lipoxygenase, however this report has not been confirmed.

As with the LTs, the EETs are substrates for glutathione conjugation, 14(15)-EET proved to be by far the best substrate (Spearman et al., 1985). The physiological relevance of such conversions remains unsure. The products so formed may have additional biological roles or alternatively GSH-conjugation may simply act as a deactivation pathway.

Lipid epoxides may be found esterified in cellular stores. Sevanian et al., (1978) demonstrated the presence of epoxides esterified to triglycerides, cholesterol and phospholipids of rat lung. However the appearance of these epoxides was believed to be through autoxidation, although the phospholipid fraction was not increased by exposure to NO_2.

GC-MS analysis of tissue samples from rat liver identified the epoxyeicosatrienoates as components of cellular phospholipids (Capdevila at al., 1984). Whether the EETs were derived from free AA and later esterified with membrane phospholipids or the AA was epoxygenated whilst esterified to the phospholipids was not determined.

1.6: Biological activities of the epoxyarachidonates

The EETs have been reported to induce a range of biological responses including inhibition of platelet aggregation and cyclo-oxygenase activity (Fitzpatrick et al., 1986, 1987; Oliw & Benthin, 1985), alteration of hormone release (Negro-Vilar et al., 1985; Capdevila et al., 1983; Turk et al., 1985; Falck et al., 1983; Grandsaert et al., 1986; Cashman et al., 1987; Snyder et al., 1983) and mobilization of Ca^{2+} (Snyder et al., 1986; Kutsky et al., 1983). The EETs have also been shown to inhibit (Na^{+}+K^{+})ATPase (Schwartzman et al., 1985) and reduce vasular resistance in the rat intestinal microcirculation (Procter et al., 1987).

Whilst investigating the fatty acid cyclo-oxygenase metabolism of 5(6)-EET, Oliw and Benthin (1985) noted that 14(15)-EET actually inhibited the enzyme. Although the potency of 14(15)-EET was equal to that of ETYA, endogenous levels of the EET would not be sufficient to act as an inhibitor. Fitzpatrick and co-workers (1986, 1987) later reported that in addition to 5(6)-EET being a substrate for fatty acid cyclo-oxygenase, 8(9)-EET and 14(15)-EET were inhibitors of the purified enzyme at micromolar concentrations. 11(12)-EET was shown to be inactive both as a substrate and an inhibitor. More detailed investigation of the inhibitory effects of the 14(15)-EET uncovered a stereospecificity. As with many other naturally occuring enantiomeric pairs the

dextrorotatory isomer (14R,15S-*cis*-EET) was the more potent. The inhibition of cyclo-oxygenase was also evident for the enzyme within intact platelets. Inhibition of platelet aggregation however differed in profile to cyclo-oxygenase inhibition. 8(9)-, 11(12)- and 14(15)-EETs were all found to inhibit platelet aggregation at micromolar concentrations. In contrast to enzyme inhibition results the inhibitory activity was not stereospecific. The anti-aggregatory activity of 5(6)-EET was not investigated.

A series of papers from the University of Texas Health Science Centre described the effects of EETs on the release of hormones. EET involvement in luteinizing hormone-releasing hormone (LHRH) stimulated luteinizing hormone (LH) release from anterior pituitary cells was described by Snyder et al., (1983). Cyclo-oxygenase and lipoxygenase enzyme inhibition by indomethacin and nordihydroguaiaretic acid (NDGA) respectively had no effect on LHRH-stimulated LH release whilst 5,8,11,14-eicosatetraynoic acid (ETYA) produced inhibition. ETYA effectively inhibits AA metabolism by the three pathways presently identified. In addition, the exposure of anterior pituitary cells to any of the four EETs induced LH release, the 5(6)-EET being the most potent. 5(6)-EET-induced LH release was shown to be greater than that induced by 10nM LHRH, however the concentration of 5(6)-EET utilised was greater than would be found *in vivo*. Although the EETs were capable of inducing LH

release the actual mechanism and indeed the final AA metabolite involved was not elucidated in the reported work.

In addition to stimulating LH release, 5(6)-EET was later reported to simultaneously induce Ca^{2+} efflux in anterior pituitary cells (Snyder et al., 1986). Indeed 5(6)-EET was found to mimic LHRH in stimulating LH release and the efflux of Ca^{2+}. However it was not verified whether the Ca^{2+} was mobilised from internal stores or simply leaked in through the cellular membrane as a result of some non-specific 5(6)-EET effect.

5(6)-EET-induced loss of Ca^{2+} from rat aortic smooth muscle microsomes and from rat liver microsomes was reported by Kutsky et al., (1983). In addition 14(15)-EET was found to decrease active Ca^{2+} uptake whilst passive Ca^{2+} binding was unaffected. The DHETs were inactive at uM concentrations. Furthermore, Ca^{2+} loss by canine aortic microsomes was increased by micromolar concentration of 5(6)- and 11(12)-EETs. No effect on Ca^{2+} release was observed for the DHETs and 8(9)-EET in aortic microsomes.

A report by Fishman et al., (1976) indicating a cytochrome P_{450} mono-oxygenase activity in the hypothalamus and the discovery of AA-metabolism by P_{450} enzymes (Oliw et al., 1981, 1982; Capdevila et al., 1981a) provoked an investigation by Capdevila et al., (1983) on the possible role of such AA-metabolites in somatostatin (SRIF) release. Absolute evidence that the

hypothalamic cytochrome P_{450} mono-oxygenase synthesised the EETs *in vivo* eluded examinations by Capdevila and his colleagues. However incubation of cells from the median eminence (ME) with AA induced SRIF release which was attenuated in the presence of ETYA but not indomethacin. Indeed the addition of indomethacin to the incubation medium produced a small increase in SRIF release. Furthermore hypothalamic microsomal fractions when incubated with AA in the presence of NADPH produced several polar compounds including 5(6)-EET and its corresponding diol. Both 5(6)-EET and 5(6)-DHET stimulated the release of SRIF from the ME *in vitro*, the epoxide being the more potent. Synthetic 14(15)-EET and its corresponding diol were ineffective in stimulating SRIF release in the same preparation. From their results Capdevila et al. proposed that AA metabolites by different pathways may be involved in the regulation of hypothalamic hormonal release.

Previous reports by Gillespie et al., (1972) and by Desai and Raghavan (1982) indicated an *in vivo* stimulation by PGs of oxytocin release from the pituitary gland. Arginine vasopressin (AVP) release from the pituitary had also been shown to be stimulated by PGs (Vilhardt & Hedqvist, 1970; Yamamoto et al., 1976). Negro-Vilar et al., (1985) therefore investigated the possible role of the EETs and HETEs as well as the PGs in the release of oxytocin and AVP. The inhibition of AA-stimulated hormone release by ETYA indicated

AA-metabolites were exerting an influence rather than the parent AA. The production of the EETs by microsomal fractions from the neural lobe of the rat pituitary was demonstrated. 8(9)-, 11(12)- and 14(15)-EETs were primarily produced, 5(6)-EET only appearing in trace amounts. Stimulation of oxytocin and AVP release from the neural lobe by the EETs followed the same pattern as their synthesis; 8(9)-, 11(12)- and 14(15)-EET being active whilst 5(6)-EET was only weakly stimulatory. Furthermore 11(12)-EET was found to favour stimulation of AVP release whilst 8(9)-EET favoured the stimulation of oxytocin release. The effect of the EETs however was found to be of less importance than that of the PG tested (PGE_2). Of the lipoxygenase products tested, 5-HETE was found to be without effect and 12-HETE only stimulated AVP release.

The EETs have also been shown to be active in isolated rat pancreatic islets *in vitro* (Falck et al., 1983). Arginine-induced stimulation of glucagon and insulin release was found to be inhibited by both metyrapone and ETYA. Similar results were obtained with glucose-induced stimulation of insulin release. The incubation of AA with isolated pancreatic islets increased the release of insulin and to a much lesser extent the release of glucagon. Both effects were again inhibited by ETYA. Incubation of the individual EETs with islet cells disclosed that only 5(6)-EET significantly stimulated insulin release whilst 8(9)-, 11(12)- and 14(15)-EET

stimulated glucagon release to increasing amounts. The stimulatory effects of the EETs were also found to be dose-dependent in the concentration range 10^{-8} to 10^{-5}M. Incubation of 5-HETE or 12-HETE with isolated islet produced an increase in glucagon secretion only. Unfortunately Falck et al. were unable to demonstrate epoxygenation of AA by the isolated rat pancreatic islets.

A later report by Turk et al., (1985) however gave a contrasting opinion as to the actions of the EETs on pancreatic islet cells. ETYA and NDGA, both lipoxygenase inhibitors, were found to inhibit glucose-induced insulin secretion from islet cells whilst not inhibiting the mono-oxygenase enzyme. Furthermore Turk et al. failed to detect any EET stimulation of insulin secretion or indeed any synthesis of the EETs by isolated islets or microsomal fractions of islets. Although a significant 5(6)-EET stimulation of pancreatic islet cells had been shown by Falck and colleagues the final insulin production was still only small in comparison to that during glucose stimulation. A role for 12-HETE was favoured by Turk et al. in the glucose-induced stimulation of pancreatic islet cells. The inhibition seen with 12-HETE by Falck et al. was questioned in their report.

Grandsaert et al. (1986) described 5(6)-EET stimulation of prolactin release from rat GH_3 anterior pituitary tumor cells. This work was later confirmed by

Cashman et al. (1987). Utilising rat anterior pituitary tumour cells (GH3 cells) the authors demonstrated a 3 to 4-fold increase in secretion stimulated by 5(6)-EET. Although 14(15)-EET and 5-HETE also stimulated prolactin secretion it was to a lesser degree than 5(6)-EET. Unfortunately a response to AA was not obtained.

Schwartzman et al., (1985) reported the presence of a cytochrome P_{450} mono-oxygenase system in the medullary thick ascending loop of Henle (mTALH). The two main metabolites of this enzyme were 5(6)-EET and 11(12)-DHET, both of which were found to be biologically active. The formation of the metabolites was stimulated by arginine vasopressin and salmon calcitonin. 5(6)-EET proved to be a potent relaxant of rabbit pulmonary artery rings precontracted with phenylephrine. 11(12)-DHET was active as an inhibitor of $(Na^+ + K^+)$ATPase.

Of the four cytochrome P_{450}-AA metabolites the 5(6)-EET proved to be the most potent vasodilator of the intestinal microcirculation in rats (Procter et al., 1987). Whilst 8(9)- and 11(12)-EET also demonstrated vasodilator activity, but to a lesser extent than 5(6)-EET, 14(15)-EET failed to evoke a response. Although the EETs had vasoactivity, *in vivo* synthesis could not be demonstrated. Application of AA to the serosa did induce vasodilation but this could be almost completely inhibited by cyclo-oxygenase inhibitors. However AA application to the mucosal surface in the presence of bile induced vasodilation that was predominantly blocked

by mono-oxygenase inhibitors. The response achieved by mucosal application of AA was smaller than that observed with serosal application.

The biological activities of the EETs are summarized in table 1.1.

1.7: Objectives

The primary objectives of this project were to establish a reliable EET synthesis and test the products for pharmacological activity in cardiovascular and respiratory preparations. In addition, all four EET-isomers were to be tested for biological activity in isolated platelets. Attention would then be turned towards determining a mechanism of action for the EETs and finally demonstrating their synthesis in the systems examined.

TABLE 1.1
Summary of the biological effects of the epoxyeicosatrienoic acids.

Source Reference Tissue	Tissue Response 5(6)-EET	8(9)-EET	11(12)-EET	14(15)-EET
Capdevila et al. 1983 somatostatin release	stimulation			inactive
Falck et al. 1983 insulin release glucagon release	stimulation inactive	inactive stimulation	inactive stimulation	inactive stimulation
Snyder et al. 1983 LH release	stimulation	stimulation	stimulation	stimulation
Kutsky et al. 1983 active Ca^{2+} uptake passive Ca^{2+} binding Ca^{2+} release	increased	no effect	increased	reduction no effect increased
Oliw and Benthin 1985 cyclo-oxygenase				inhibition
Negro-Vilar et al. 1985 oxytocin and AVP release	weak stimulation	oxytocin stimulation	AVP stimulation	stimulation
Turk et al. 1985 insulin release	inactive			
Schwartzman et al. 1985 pulmonary artery	relaxation			
Fitzpatrick et al.'86/7 platelets cyclo-oxygenase	substrate	anti- aggregatory inhibition	anti- aggregatory inactive	anti- aggregatory inhibition
Snyder et al. 1986 Ca^{2+} efflux (ant. pit.)	stimulation			
Grandsaert et al., 1986 prolactin release	stimulation			
Cashman et al. 1987 prolactin release	stimulation			stimulation
Procter et al. 1987 intestinal vasculature	relaxation	relaxation	relaxation	no effect

CHAPTER 2: EPOXIDE SYNTHESIS AND SEPARATION

2.1: INTRODUCTION

Several methods of selective and non-selective EET synthesis have been reported. Non-selective epoxidation of AA yielding a mixture of all four EET-isomers has been described by Oliw et al. (1982), based on the oxidation of polyunsaturated fatty acids by m-chloroperoxybenzoic acid (Chung & Scott, 1974). Selective methods of synthesising the different regio-isomers have also been described. 5(6)-EET and 14(15)-EET were synthesised from the parent fatty acid, AA (Corey et al., 1979). 8(9)-EET was sythesised from the 5(6)-isomer (Falck & Manna, 1982) and 11(12)-EET was synthesised from the 14(15)-isomer Corey et al., 1980).

The well established method described by Oliw et al. (1982) was initially adopted for the non-selective synthesis of all four EETs. Separation of the individual EETs from the reaction mixture was accomplished using thin layer chromatography (Chung & Scott, 1974). Routine HPLC separation of the EETs as described by Oliw et al. (1982) was also evaluated.

The poor yield of 5(6)-isomer obtained by non-selective epoxidation necessitated the evaluation of selective 5(6)-EET synthesis according to the method of Corey et al. (1979).

2.2: METHODS

2.2.1: Synthesis & Separation of the EETs

Non-selective epoxidation of 1-^{14}C radio-labelled AA by m-chloroperoxybenzoic acid (m-CPBA) in dichloromethane was achieved utilising a modification of the protocol described by Oliw et al. (1982). AA and m-CPBA were reacted in molar ratios of 1:1, 1:1.5, 1:2, 1:5 and 1:10. The products were separated by TLC using a solvent of ethylacetate/hexane/acetic acid 35:65:0.1 (Oliw et al., 1982) and visualised by iodine staining. The reaction mixture ratio (AA:mCPBA) giving optimum AA epoxidation without excessive product breakdown was adopted for routine EET synthesis.

Other TLC solvent systems were evaluated due to poor resolution of the four epoxide isomers obtained using the system described by Oliw et al. (1982). Normal phase silica gel TLC plates (plate thickness 0.25mm, particle size 0.25mm, pore size 60A) were used throughout for the separation of the EETs. Solvent systems investigated included ethylacetate/hexane/acetic acid 40:20:1, hexane/ether 4:1 (Corey et al., 1979), ethylacetate/ hexane/acetic acid 30:70:0.1, ethylacetate/hexane/acetic acid 35:65:0.1 (Oliw et al., 1982) and ether/hexane/ acetic acid 30:70:1 (Chung & Scott 1974). The solvent system giving greatest resolution of the four EETs and any unchanged AA or breakdown products was adopted for

routine EET separation.

500mg of AA was oxidised by 1.5 molar equivalents of mCPBA in a final reaction volume of 25ml of dichloro-methane for 17hrs at 20°C under nitrogen.

Using the chosen solvent system of ether/hexane/acetic acid (30:70:1), TLC plates (loaded with 0.2mg of reaction mixture per lane) were developed twice to 17cm. After drying, the EETs on the end two lanes on each plate were visualised using iodine. Corresponding epoxide bands were removed from the plates by scraping and the EETs eluted from the silica with three serial 10ml aliquots of dry ether. Once eluted the epoxides were stored in liquid nitrogen, the pooled eluates evaporated to dryness under vacuum using a Gyro-Vac centrifugal evaporator before being reconstituted in 0.4ml of dry methanol. Methanolic solutions of the EETs were stored under nitrogen until required.

The final yields of the four EETs were assessed by liquid scintillation counting using an LKB Rackbeta 1215 counter, the count being converted to EET content by comparison to the initial reaction mixture count.

2.2.2: Identification of the EETs

The identities of the four EETs was confirmed using GC-MS and 2D-TLC. For GC-MS analysis, aliquots of the four epoxides were methylated at the carboxylic acid group using an ethereal solution of diazomethane (see below). The methylated EETs were then evaporated to dryness and reconstituted in HPLC-grade ethylacetate. Positive identification was obtained by GC-MS using a Hewlett-Packard 59770C MS ChemStation. 2ul samples were injected onto an OV-1, 12m capillary column. The column temperature was ramped from 55°C to 270°C increasing by 30°C/minute. Eluates were ionised by electron impact using an electron energy of 70eV, the search parameters set between 50 and 400 atomic mass units. In the case of 2D-TLC a mixture of non-methylated EETs was loaded onto a plain silica gel plate (20cm^2, plate thickness 0.25mm, particle size 0.25mm, pore size 60A) and developed over 17cm in the first dimension using a solvent of ether/hexane/acetic acid (30:70:1). After drying the plate was developed over the second dimension with a solvent of ethylacetate/hexane/acetic acid (35:65:0.1). The position of the EETs was visualised by iodine staining.

Synthesis of diazomethane

6g of potassium hydroxide in 10ml of water was placed in a 100ml distilling flask together with 35ml of

diethylether and 10ml of water. The flask was fitted with a dropping funnel and a downward condenser which emptied into a collecting flask cooled on ice. The solution was warmed to a temperature of 70 to 75°C in a water bath. At the first sign of ether being distilled a solution of 21.5g of *N*-methyl-*N*-nitrosotoluene-*p*-sulphonamide in 125ml of ether, already in the dropping funnel, was introduced to the distillation flask dropwise. The rate of addition of the nitrosoamide was adjusted to equal the rate of distillation. Once all the nitrosoamide had passed into the distillation flask, further aliquots of ether were added through the dropping funnel until the distillate became colourless.

Using a blunt pasteur pipette, the ethereal solution of diazomethane was transferred in molar excess to the epoxide solutions. Excess diazomethane was neutralised by the dropwise addition of glacial acetic acid until no further effervescence was observed.

2.2.3: Selective 5(6)-EET synthesis

The method of selective epoxidation of AA at the 5,6 position as described by Corey et al. (1979) was considered. Briefly, arachidonic acid in tetrahydrofuran water (AA:THF-water, 1.7:1) was reacted with 5 molar equivalents of potassium bicarbonate and 8 molar equivalents of potassium triiodide for 3 days at 4°C. Following extraction the products were further reacted in THF-water (3:2) with excess 0.2N lithium hydroxide for 3 hours at 25°C. Extraction and evaporation yielded the final product which could be reconstituted in dry methanol.

Potassium triiodide was synthesised as described by Foote & Chalker (1908). Briefly, a stoichiometric amount of iodine was slowly added to a solution of potassium diiodide with constant stirring at 60°C. The final solution was then cooled to 0°C.

2.2.4: HPLC Separation

Reverse-phase HPLC separation of the EETs was performed using an analytical column (150mm length x 7mm internal diameter) packed with Lichroprep RP18 ODS, 25-40um. 0.1mg samples underwent separation using a mobile phase of 73% methanol in water with 0.2% acetic acid (Oliw et al., 1982) at a flow rate of 2ml/minute driven by an LDC Constametric III pump. Elution of

conjugated products was monitored using an LDC Spectromonitor III measuring UV absorbance at 320nm. The eluate was collected in 2ml fractions using an LKB Helirac 2212, and the position of the peaks determined by liquid scintillation counting.

2.3: RESULTS

2.3.1: Synthesis & Separation of the EETs

The optimal molar ratio of AA:mCPBA was found to be 1:1.5. At this ratio some AA remained with only slight product oxidation taking place. Lower ratios gave only poor yields of EETs whilst higher ratios showed excessive subsequent oxidation of the products (Figure 2.1).

Of the solvent systems evaluated for routine EET separation only ether/hexane/acetic acid (30:70:1) gave complete separation of the four EETs. Using ethylacetate/ hexane/acetic acid (35:65:0.1) or ethylacetate/hexane/ acetic acid (30:70:0.1), the 5(6)- and 8(9)-EETs were unresolved. With ethylacetate/hexane/acetic acid (40:20:1) as solvent, all the products migrated with AA at the solvent front. In the non-acidified solvent, hexane/ ether (4:1) all the products and AA were retained by the silica at the origin (Figure 2.2).

A representative set of Rf values for the EETs, relative to AA (solvent ether/hexane/acetic acid 30:70:1), from one separation were 0.854, 0.797, 0.577 and 0.707 for the 14(15)-, 11(12)-, 8(9)- and 5(6)-EETs respectively. The final ratio following extractive isolation of 14(15)-, 11(12)-, 8(9)- and 5(6)-EET was 79:37:33:4 respectively. Of the AA initially reacted with mCPBA approximately 40% was recovered as EET.

FIGURE 2.1

Thin-layer chromatographic separation (solvent: ethylacetate/hexane/acetic acid 35:65:0.1) of EETs and AA following epoxidation of AA with increasing molar ratios of m-chloroperoxybenzoic acid (m-CPBA:AA). A ratio of 1.5:1 gave the best yield with least breakdown of the EETs. Abbreviations: AA, arachidonic acid; EETs, epoxy-eicosatrienoic acids.

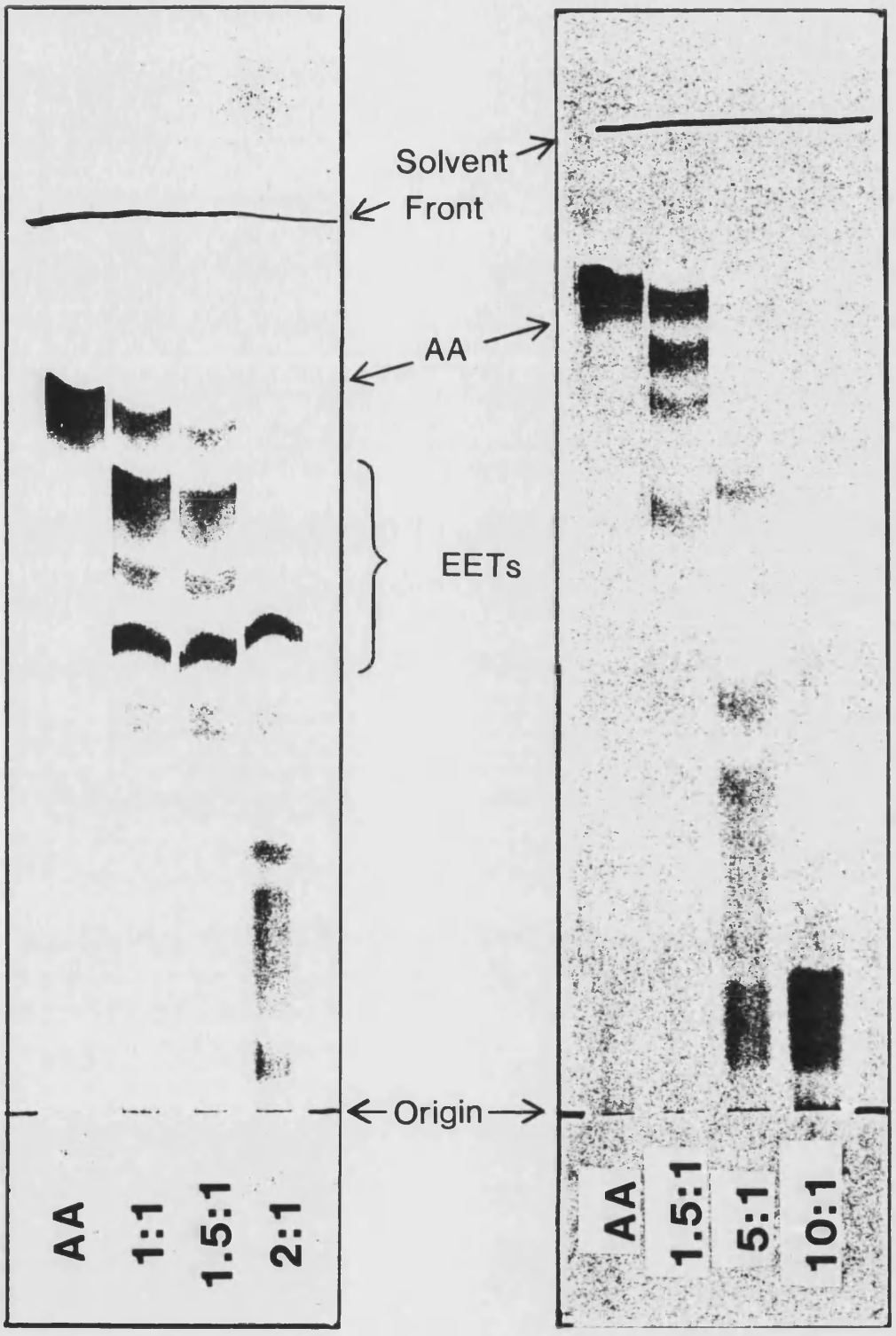

Solvent Front
AA
EETs
Origin
AA
1:1
1.5:1
2:1
AA
1.5:1
5:1
10:1

FIGURE 2.2

Separation of AA and EETs by different solvent systems using thin layer chromatography. 0.2mg of ^{14}C radio-labelled reaction mixture was separated using different solvents. A solvent of ether/hexane/acetic acid (30:70:1) gave the best separation. Abbreviations: EtOAc, ethylacetate; Hex, hexane; GAA, glacial acetic acid; AA, arachidonic acid; EET, epoxyeicosatrienoic acid.

EtOAc/Hex/GAA 40:20:1
Hex/Ether 4:1
EtOAc/Hex/GAA 30:70:0.1
EtOAc/Hex/GAA 35:65:0.1
Solvent Front
Ether/Hex/GAA 30:70:1
AA
14(15)-EET
11(12)-EET
5(6)-EET
8(9)-EET
Origin

2.3.2: Identification of the EETs

Mass spectrographic analysis of the four methylated compounds, together with 2D-TLC of the separated compound substantiated the identities as the 5(6)-, 8(9)-, 11(12)- and 14(15)-EETs. The GC retention times of the methylated EETs were 8.93, 8.90, 8.83 and 8.85 minutes for the 14(15)-, 11(12)-, 8(9)- and 5(6)-isomers respectively.

The more important mass ions indicative of the EETs present in the MS traces have mass/charge values of 303, 263*, 245, 235, 234 and 220 for 14(15)-EET (Fig. 2.3), and 303, 290, 263, 245, 223*, 207 and 205 for 11(12)-EET (Fig. 2.4). Important mass ions for 8(9)-EET were detected at mass/charge values of 245, 193*, 175 and 165 (Fig. 2.5), and important mass ions for 5(6)-EET were detected at 245, 233*, 215 and 143* (Fig. 2.6). The mass ions resulting from cleavage around the epoxide bridge are superscripted with an asterisk. Table A3.1 shows the mass/charge values of all the ions detected for each EET together with their abundances. The M^+ ion (mass/charge 334) was not detected in any of the EET spectra.

The identities of the EET bands following separation using ether/hexane/acetic acid (30:70:1) were confirmed by the second development using ethyacetate/hexane/acetic acid (35:65:0.1). The relative positions of the EETs using the second solvent are known from the work of Oliw et al., (1981). From these the relative positions of the bands following separation with ether/hexane/acetic acid

were determined. As the 5(6)- and 8(9)-EETs were not resolved utilising ethylacetate/hexane/acetic acid the final identification of these two EETs was achieved by GC-MS.

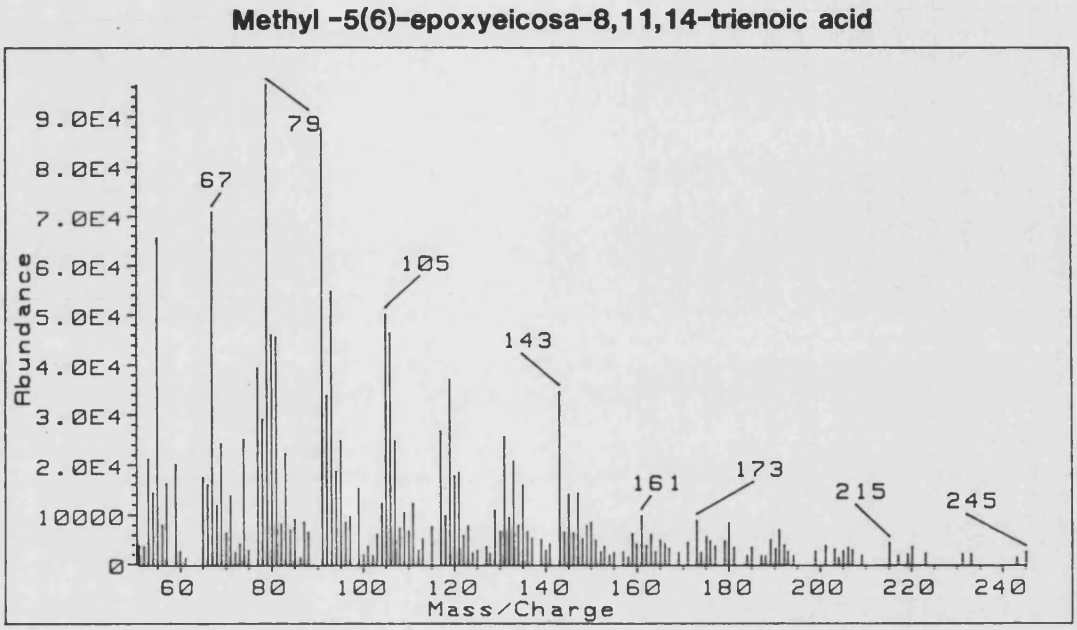


FIGURE 2.3
MS of methyl-5(6)-EET. Mass ion 233 results from the cleavage of the C4-C5 bond and mass ion 143 results from the cleavage of C6-C7 bond around the epoxide bridge.

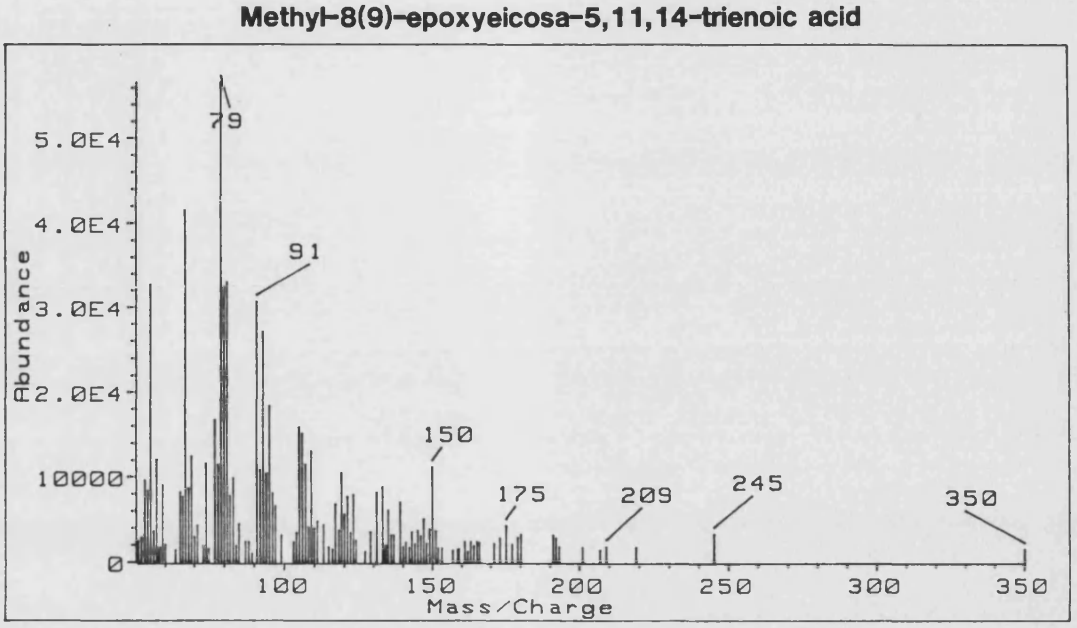


FIGURE 2.4
MS of methyl-8(9)-EET. Mass ion 193 results from the cleavage of the C7-C8 bond.

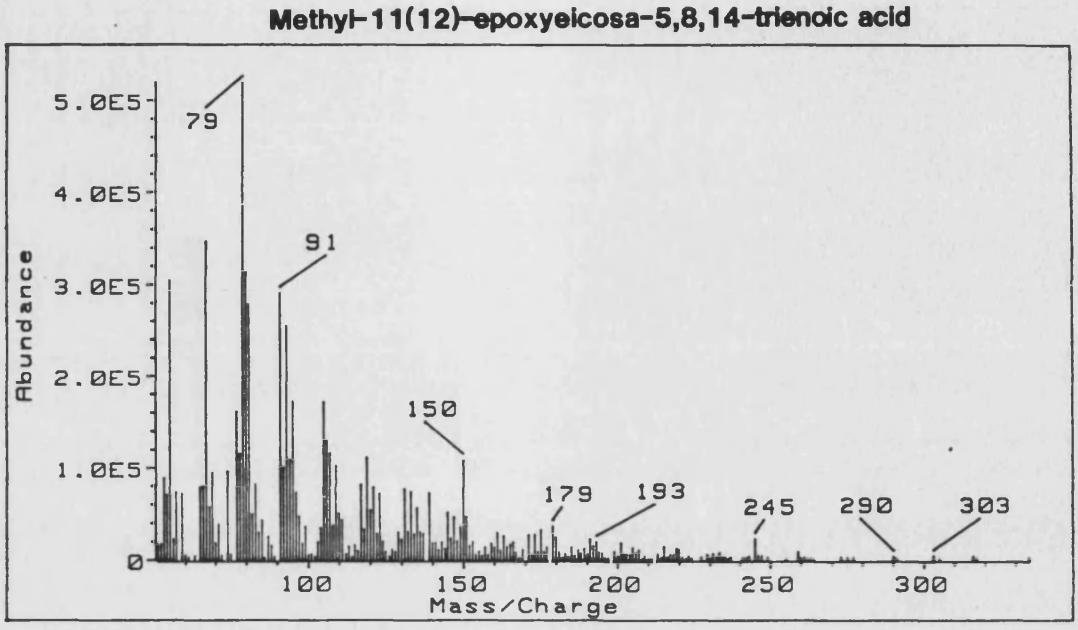


FIGURE 2.5
MS of methyl-11(12)-EET. Mass ion 223 results from the cleavage of the C12-C13 bond.

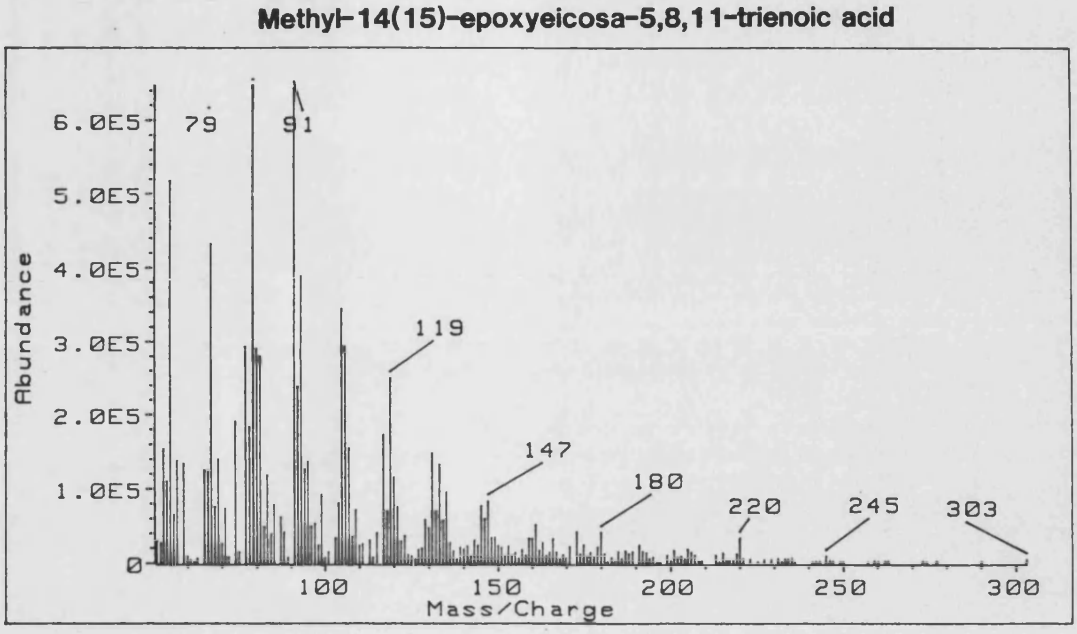


FIGURE 2.6
MS of methyl-14(15)-EET. Mass ion 263 resulted from the cleavage of the C15-C16 bond.

2.3.3: Selective 5(6)-EET synthesis

The products from selective epoxidation of AA were examined using TLC with a solvent of ether/hexane/acetic acid (30:70:1). Previously synthesised 5(6)-EET and AA were used as standards. Although some unreacted AA remained no 5(6)-EET was detected in the mixture.

2.3.4: HPLC Separation

4 minutes after sample injection onto the column a non-radiolabelled peak with high UV absorbance was detected corresponding to 3-chlorobenzoic acid, the polar breakdown product of the oxidising agent. The appearance of this peak was used as a marker to initiate collection of the eluate. Radiolabelled peaks were collected after 30ml, 50ml, 60ml, 122ml, 144ml and 198ml. Although none of the eluted compounds were positively identified the peak at 198ml was substantially greater than all the rest and was presumed to be residual AA. Only poor resolution of the peaks at 50ml and 60ml and also at 122ml and 144ml was achieved.

2.4: DISCUSSION

2.4.1: Synthesis and Separation of the EETs

The ratio of EETs produced by non-selective epoxidation of AA (79:37:33:4) differed from that achieved by Oliw et al. (1982). The much reduced yield of the 5(6)-isomer is probably due to the harsher separation procedure followed here. The double TLC development required for optimal separation was probably instrumental in the poor yield due to the increased exposure of the EETs to the atmosphere and possibly water. This resulted in the loss of some of the synthesised EETs, especially the 5(6)-regioisomer.

The low conversion of AA to EET could have been improved by reacting an increased amount of mCPBA, however a compromise was required between low conversion and the possible loss of EETs by further epoxidation. The increased molar proportion of mCPBA found to be suitable here in comparison to that used by Oliw et al. (1982) was probably due to chemical degradation of the oxidising agent during storage.

2.4.2: Identification of the EETs

As no standard EETs were readily available for GC-MS analysis, the spectra obtained for the reaction products could only be compared with those published by Oliw et

al. (1982). However, the two sets of spectra were sufficiently similar to identify the products as the EETs. The 5(6)-EET was instantly recognisable by the characteristic peak at m/s143. The other isomers were deduced by carefully examining a host of individual MS characteristics. The absence of a mass ion at m/s334 was undoubtedly due to the harsh ionisation technique utilised by this system. The identification of the compounds separated by TLC was confirmed by 2D-TLC.

2.4.3: Selective 5(6)-EET Synthesis and HPLC Separation

The failure to synthesise 5(6)-EET by selective epoxidation was probably due to the difficulties and uncertainties encountered in preparing the potassium triiodide. The chemical composition of the KI_3 solution prepared was not confirmed so the efficiency of the initial reaction was unknown. Further attempts to overcome these difficulties were not undertaken due to time restraints.

Incomplete resolution of the EETs achieved by HPLC could have been rectified by compositional manipulation of the mobile phase. However, as the maximum sample size which could be applied to the column at any one time was limited, further scrutiny of the system was not justifiable. To permit sufficient product to be separated in a reasonable time would have required regular use of a preparative HPLC column, access to which was not

available.

Although not ideal for the large scale production of EETs, non-selective epoxidation of AA and separation of the products by TLC provided a sufficient supply to meet experimental requirements.

CHAPTER 3: SMOOTH MUSCLE PHARMACOLOGY OF THE EETs

3.1: INTRODUCTION

The basic structure of arteries consists of three distinct layers: the tunica adventitia, the tunica media and the innermost layer the tunica intima. In addition there is a single layer of endothelial cells lining the vessel lumen. Vascular smooth muscle accounts for the tunica media, the fibres being arranged circularly around the lumen. Vascular resistance is determined by smooth muscle tone, especially in the arterioles. The degree of contracture of vascular smooth muscle is controlled both neuronally and by local hormones. Noradrenergic activation results in vasocontriction (except in skeletal vessels where cholinergic activation induces relaxation). 'Local hormones', including AA-metabolites, induce both contraction and relaxation (see below).

The endothelial cell lining, invariably only one cell thick, prevents contact between blood cells (including platelets) and the underlying tissue thereby preventing thrombus formation. In addition to its anti-thrombotic role, the endothelial lining has recently been implicated in the control of vascular tone by the release of endothelium derived relaxing factors (reviews: Furchgott, 1983, 1984; DeFeudis, 1985).

Respiratory smooth muscle tone is also controlled by both neuronal and hormonal stimuli. Parasympathetic

release of ACh is resposible for contraction, whilst pre-synaptic α-adrenoceptor stimulation by sympathetic innervation reduces the para-sympathetic control. β_2-adrenoceptors located on the smooth muscle surface may be stimulated by circulating adrenaline. Metabolites of AA are also known to induce smooth muscle responses (see below).

Both cAMP and cGMP have been identified as second messengers in the relaxant responses of smooth muscle. Stimuli responsible for the activation of adenylate cyclase are now known to be different from those which activate guanylate cyclase, although the physiological effects of both processes have many similarities.

Agents inducing relaxation in various smooth muscles, including vascular and respiratory preparations, are known to stimulate an increase in cyclic GMP levels which correlates well with observed relaxation. Furthermore, the elevation in cGMP preceeds smooth muscle relaxation. Amongst the relaxant agonists found to elevate cGMP levels are EDRF, the nitrovasodilators and the atrial natriuretic factors (Ignarro et al., 1986; Waldman et al., 1984; 1985).

While the precise mechanisms involved are still unknown, elevation of cGMP is accompanied by the activation of cGMP-dependent protein kinases (Forstermann et al., 1986). Activation of kinases results in the phosphorylation of several cellular proteins, the pattern

of which is consistent thoughout the range of guanylate cyclase activating agents (Rapaport et al., 1982; 1983; Draznin et al., 1983; 1986). Dephosphorylation of myosin light chain, demonstrable during smooth muscle relaxation, has also been observed as a result of cGMP synthesis suggesting the activation of phosphoprotein phosphatases (Draznin et al., 1983; 1986).

Increased levels of cGMP have also been associated with reduced cytosolic calcium levels (Johnson & Lincoln, 1985). Furthermore, elevated cGMP levels activate membrane Ca^{2+}-ATPase which could pump calcium directly from the cytosol (Popescu et al., 1985) however direct activation of Ca^{2+}-ATPase by cGMP-dependent protein kinase has not been demonstrated (Lincoln et al., 1988). Such reductions in cytosolic calcium were hypothesised as possibly leading to reduced activity of Ca^{2+}-dependent myosin light chain kinase, dephosphorylation of myosin light chain and hence relaxation (Lincoln et al., 1988).

Alternatively, calcium release from internal stores by inositol trisphosphate (Yamomoto & Breemen, 1985) may be reduced due to inhibition of phosphoinositide hydrolysis by cGMP (Lewis et al., 1988).

During periods of tonic contraction cytosolic free calcium levels are found to revert to basal levels (Bradley & Morgan, 1987). In this case increased cGMP levels have been proposed to reverse contraction by the inhibition of diacylglycerol (DAG) formation from phosphoinositides (Lewis et al., 1988). Elevated levels

of DAG are observed during prolongued smooth muscle contraction (Griendling et al., 1986) and DAG stimulation of protein kinase C is considered to be responsible for extended periods of smooth muscle contraction (Forder et al., 1985).

In vasculature, β-adrenergic stimulation (Lefkowitz et al., 1983) or exposure to PGI_2 (Ignarro & Kadowitz, 1985) stimulates adenylate cyclase which converts ATP to cAMP. Once formed, cAMP activates a cAMP-dependent protein kinase resulting in the phosphorylation of smooth muscle proteins. However the phosphorylation profile is different from the case of elevated cGMP levels. As with cGMP, the actual mechanism linking raised cyclic nucleotide levels to smooth muscle relaxation are undetermined although a similar scheme of kinase activation and protein phosphorylation are known to be associated with cAMP increases.

The biological activity of AA-metabolites in vascular and respiratory models is well documented. The responses observed, either contraction or relaxation, are dependent upon the species and tissue examined and even on the muscle tone immediately prior to exposure to eicosanoid. Prostanoid receptors with structural specificity to the individual PGs have been identified and standard nomenclature determined (e.g. DP, EP_1, EP_2, EP_3, FP, IP & TP). Receptors DP, EP_2 and IP have cAMP as their effector

pathway, whilst receptors EP_1, EP_3, FP and TP have IP_3/DAG as their effector pathway. EP_3 receptor stimulation may also mediate its effects via decreasing cAMP levels (TIPS, 1990). Of interest is the presence of EP receptor subtypes with opposing actions in guinea-pig trachea, see section 3.6.1b (Dong et al., 1986). Prostaglandins E_2, D_2 and I_2 are known to relax the aorta, whilst $PGF_{2\alpha}$, TXA_2 and the LTs induce constriction. In the guinea-pig trachea however PGE_2 is relaxant, whereas PGI_2 is without direct effect although it may oppose the effects of other constrictor compounds. Again $PGF_{2\alpha}$, TXA_2 and the LTs are constrictor (Moncada et al., 1980; Messina et al., 1976).

Epoxyeicosatrienoic acid-induced relaxation has been reported in the rat caudal artery and intestinal microvasculature (Carroll et al., 1987, 1988; Procter et al., 1987). The relaxant properties of 5(6)-EET in the caudal artery were attributed entirely to its further metabolism through cyclo-oxygenase (Carroll et al., 1987). This chapter describes EET-induced smooth muscle responses in a large blood vessel, the rat aorta, and in respiratory smooth muscle using the guinea-pig trachea. Cyclo-oxygenase and lipoxygenase involvement in EET-induced responses were also investigated, together with the role of cyclic nucleotides in EET-induced relaxation.

3.2: RAT ISOLATED AORTA

Male Wistar rats (330-370g) were killed by a blow to the head, the thoracic aorta removed and cleaned of adherent fat and connective tissue. Circular smooth muscle strips approximately 3mm wide were prepared and mounted in 10ml jacketed organ baths containing Krebs buffer at 37°C. Where required, de-endothelialisation of the aortic strips was achieved, before mounting in the organ bath, by rolling a cotton bud soaked in Krebs solution across the intimal surface several times. After mounting in the organ bath, the initial tension was adjusted to 2g and 45-60 minutes was allowed for the tissue to equilibrate.

Following cumulative dosing with PE a dose giving approximately 80% of maximal contraction was chosen to induce tissue tone. The integrity of the endothelium of each strip was ascertained using a dose of ACh known to give a maximal relaxation in strips with a preserved endothelium. Only tissues showing an 80% or greater relaxation to ACh (expressed as a percentage of maximal NaNP-induced relaxation) were assumed to have an intact endothelium. The success of de-endothelialisation of the strips was also confirmed using ACh and NaNP. Only tissues giving a full NaNP-induced response whilst showing no relaxation to ACh were utilised as denuded preparations.

Relaxant dose-response curves by both intact and

de-endothelialised rat aortic strips to the four EETs were constructed by cumulative dosing. These responses were expressed as a percenage of the maximal NaNP-induced relaxation determined at the end of each dose-response curve. In addition, the effect of pre-incubation of intact and denuded aortic strips with $3x10^{-6}$M indomethacin or $1x10^{-6}$M NDGA prior to EET addition was evaluated.

The involvement of cyclic GMP in the EET-induced responses was ascertained by pre-incubating the tissue with the selective cGMP phosphodiesterase inhibitor M&B 22,948. The effects of M&B 22,948 on PE-, ACh-, NaNP- and isoprenaline-induced responses were also investigated for control purposes.

3.3: GUINEA-PIG ISOLATED TRACHEA

Male Dunkin Hartley guinea-pigs (500-700g) were killed by a blow to the back of the head, the trachea carefully removed and placed in ice-cold Krebs solution. After removal of adherent tissue, opened rings approximately 3mm wide were prepared and mounted in 10ml jacketed organ baths under an initial tension of 3g. The Krebs solution was maintained at 37°C and gassed with 5% CO_2 / 95% O_2. The bathing fluid was changed every 20 minutes and a two hour equilibration period allowed.

The sensitivity and responsiveness of the tracheal strips to ACh was ascertained by adding the drug to the

organ bath cumulatively and recording the responses obtained. A dose of ACh giving a submaximal response (approximately 80% of maximum) was identified and used throughout the experiment to induce tone.

A parallel set of experiments were carried out using tracheal strips from the same guinea-pigs which were exposed to either $3x10^{-6}$M indomethacin or $1x10^{-6}$M NDGA throughout to inhibit cyclo-oxygenase or lipoxygenase activity respectively. A submaximal dose of ACh giving a comparable response to that in untreated tracheal strips was used to pre-contract the tissues.

EET-induced tracheal responses were examined by cumulative addition of each EET to the organ bath. A dose of NaNP known to give maximal relaxation in tracheal strips was added after the EETs to induce the maximal possible relaxation. The EET-induced relaxation was expressed as a percentage of this NaNP response.

Additions of methanolic drug solutions were kept below 3 microlitre, previous experiments indicating that such additions had no effect on tissue viability.

For statistical analysis Student's t-test for unpaired observations was used. When P was smaller than 0.05 values were considered to be significantly different.

3.4: RESULTS: RAT THORACIC AORTA

3.4.1: Control Responses

Preliminary investigations utilised male Wistar rats weighing 220-280g. Although reproducible dose-response curves to PE, ACh and NaNP were obtainable, sub-maximal PE-induced contracture was often poorly maintained. Following PE-induced contraction intact tissues commenced phasic relaxation and contraction (Fig. 3.1a). This phenomenon was observable in aorta taken from rats up to 330g in weight. In de-endothelialised preparations however these spontaneous relaxations were not observed. Above 330g the aortic preparations were found to maintain a stable sub-maximal tone to PE (Fig. 3.1b).

During tissue equilibration the basal tension of the tissue decreased to between 0.8 and 1.2g after approximately 45 minutes. PE induced a dose-dependent contraction of rat aortic strips in the range of $3x10^{-9}$ to $3x10^{-4}$M in intact strips and 10^{-9} to 10^{-4}M in denuded strips (Fig. 3.2). EC_{50} values for PE-induced contraction of intact and denuded strips were $2.5 \pm 0.1 \times 10^{-8}$M (n=17) and $7.3 \pm 0.4 \times 10^{-9}$ (n=14) respectively, representing nearly a 3 fold increase in sensitivity following de-endothelialisation. A sub-maximal dose of 10^{-7}M was used to induce a sustainable tissue tone in intact strips, and $3x10^{-8}$M in denuded preparations.

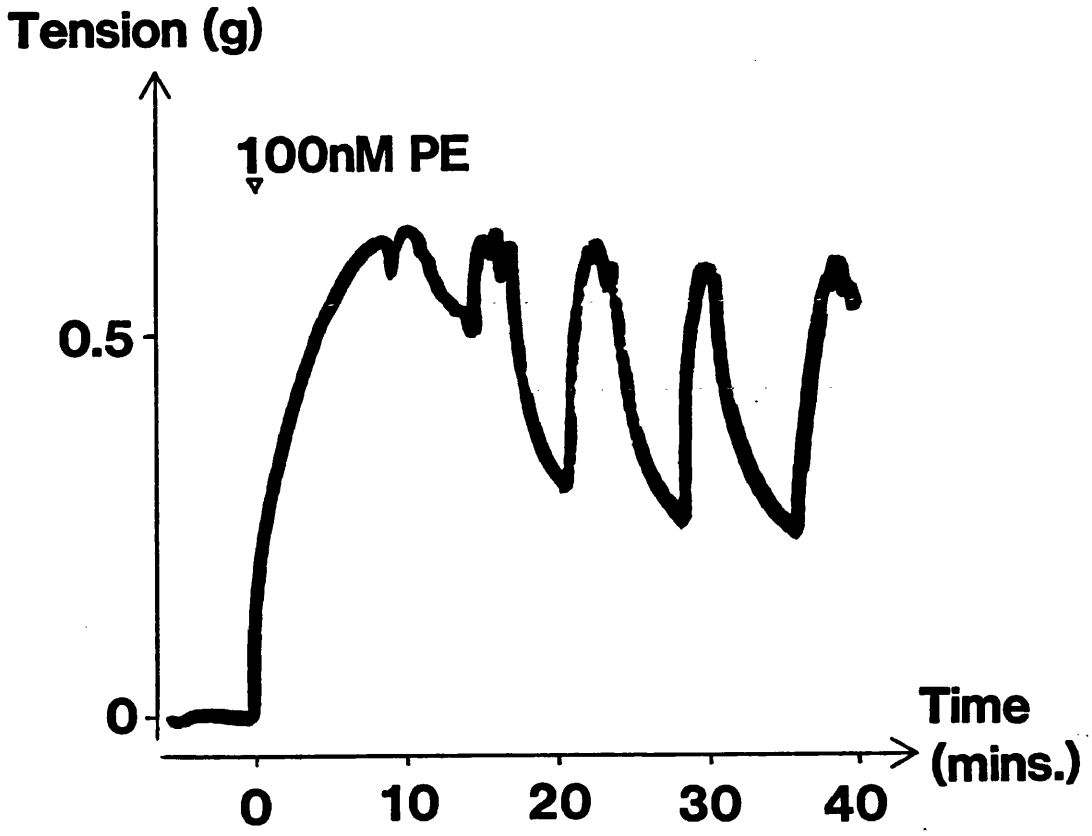


FIGURE 3.1a
Typical trace demonstrating the unstable PE-induced contracture ($1x10^{-7}M$) observed in aorta taken from rats of body weight less than 330g.

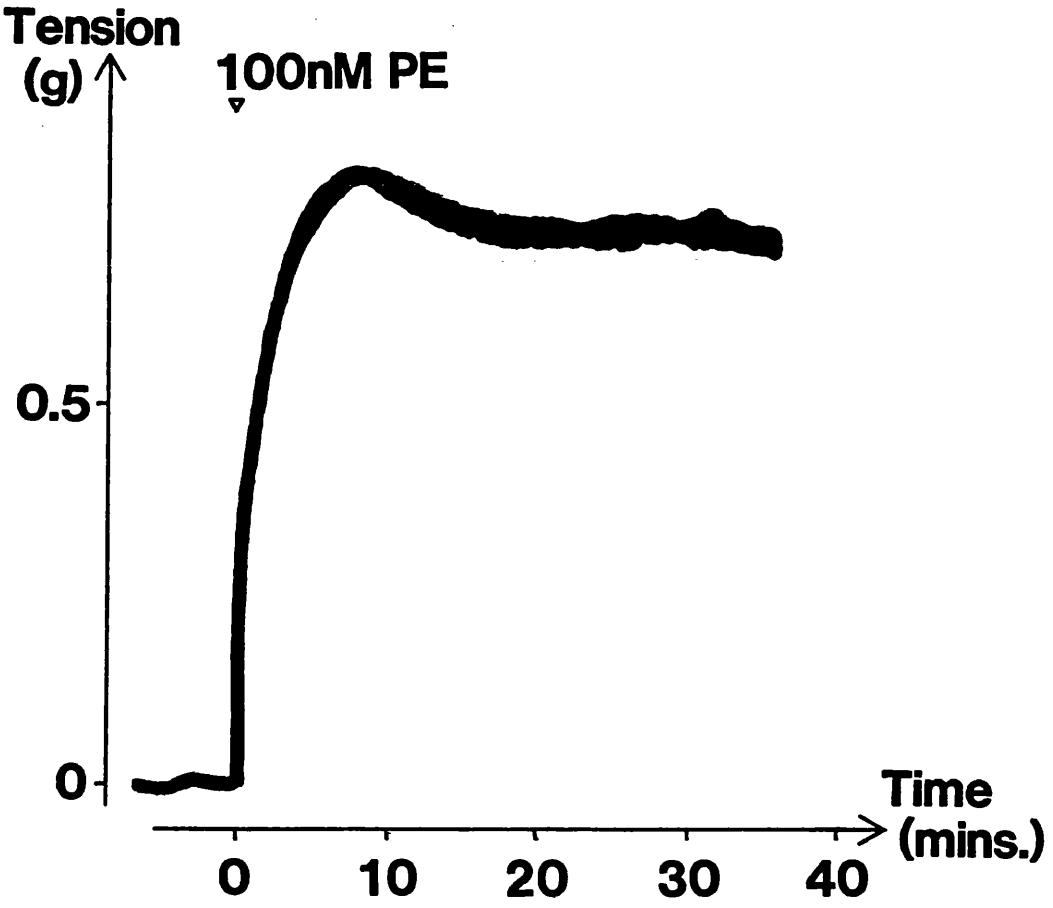


FIGURE 3.1b
Typical trace demonstrating a stable PE-induced contracture readily obtained in aorta taken from rats of body weight greater than 330g. Rats with a body weight of 350g were used routinely.

De-endothelialised aortic strips were significantly more sensitive to PE ($P<0.05$). NaNP induced relaxation in the range of 10^{-10} to 10^{-7}M with EC_{50} values of $1.6 \pm 0.1 \times 10^{-9}$M (n=15) and $1.8 \pm 0.2 \times 10^{-9}$M (n=15) for intact and denuded preparations respectively (Fig. 3.3). NaNP-induced relaxations were not significantly altered by de-endothelialisation ($P>0.50$).

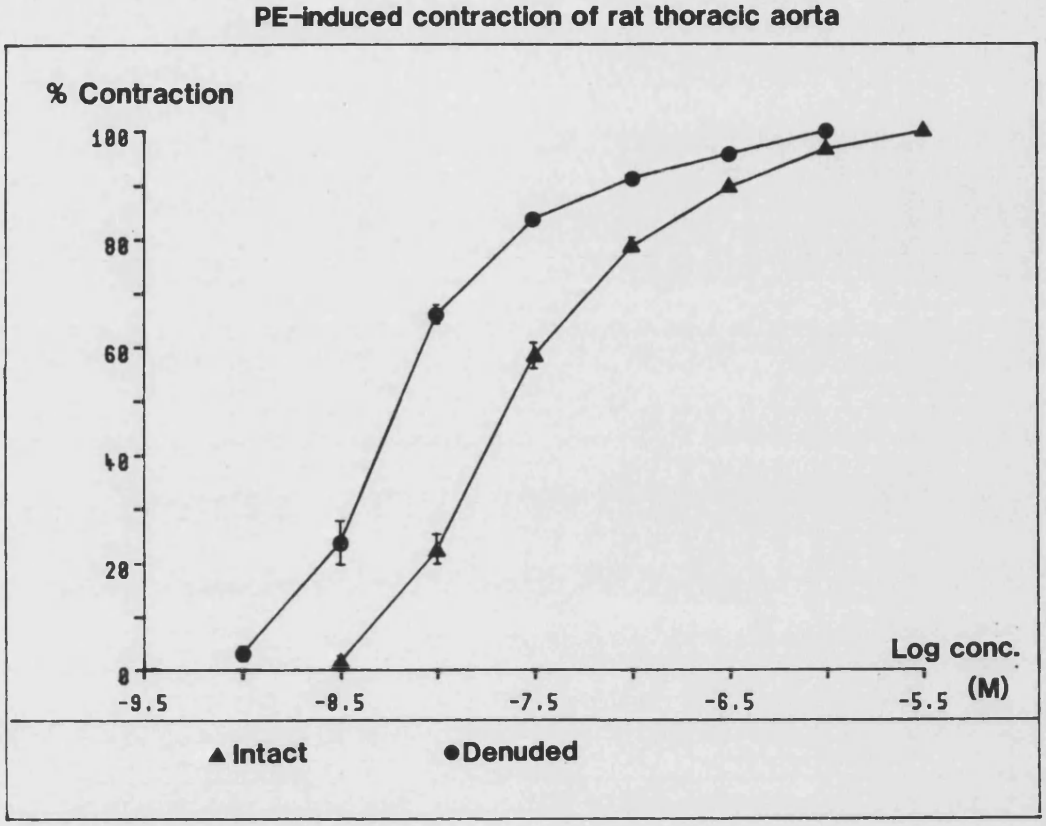


FIGURE 3.2

PE-induced contraction of rat thoracic aorta. Cumulative addition of PE induced contraction of rat thoracic aorta in the presence and absence of an intact endothelium. In intact aorta, PE induced contraction with an EC_{50} value of $2.5 \pm 0.1 \times 10^{-8}M$ (n=17). In aorta denuded of endothelial PE induced contraction with an EC_{50} value of $7.3 \pm 0.4 \times 10^{-8}M$ (n=14).

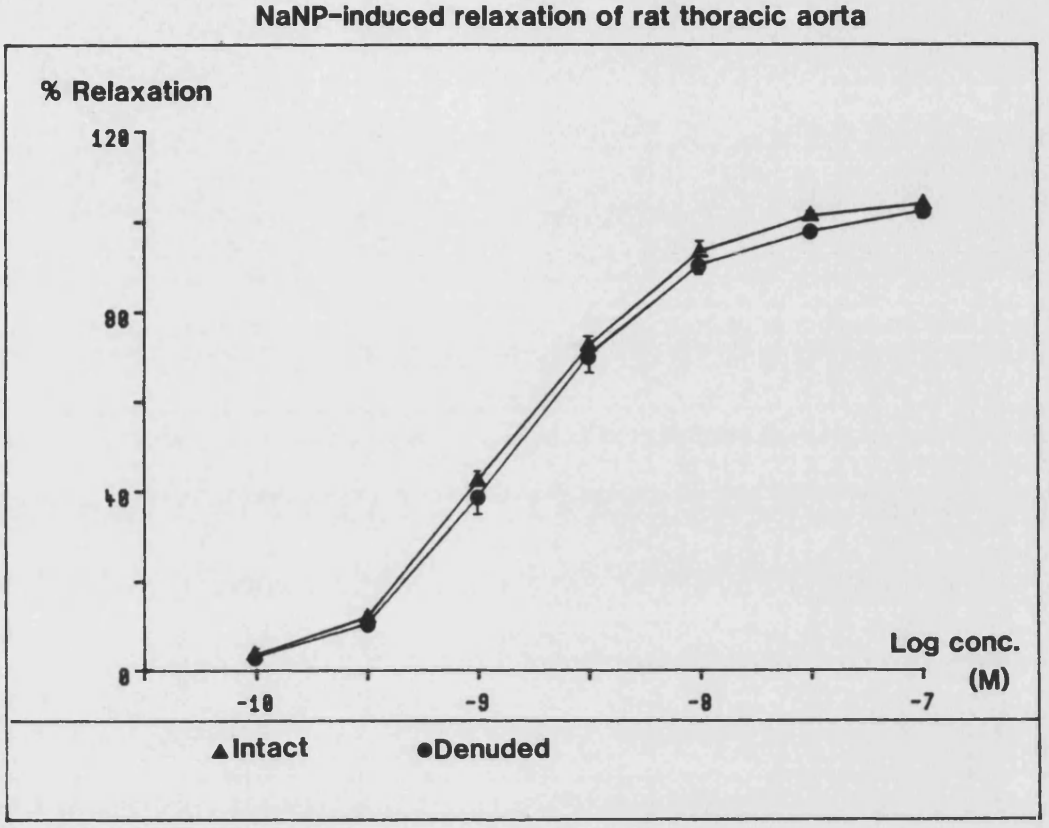


FIGURE 3.3
NaNP-induced relaxation of rat thoracic aorta. Cumulative addition of NaNP induced relaxation of rat thoracic aorta to the same degree both in the presence or absence of the endothelium. NaNP induced relaxation with EC_{50} values of $1.6 \pm 0.1 \times 10^{-9}$M (n=15) in intact aorta and $1.8 \pm 0.2 \times 10^{-9}$ (n=15) in aorta denuded of an endothelial lining.

3.4.2: EET-induced Relaxations of Rat Aorta

In intact aortic strips (Fig. 3.4), 5(6)-EET induced relaxations in the range $3x10^{-9}$ to $3x10^{-7}M$ with an EC_{50} of $3.9 \pm 0.6 \times 10^{-8}M$ (n=4). 8(9)-, 11(12)- and 14(15)-EET were roughly equipotent whilst being less potent than 5(6)-EET at inducing relaxation. 8(9)-, 11(12)- and 14(15)-EET were relaxant in the range 10^{-6} to $3x10^{-5}M$ with EC_{50} values of $5.2 \pm 0.7 \times 10^{-6}M$ (n=4), $6.7 \pm 0.2 \times 10^{-6}M$ (n=3) and $8.6 \pm 0.2 \times 10^{-6}M$ (n=3) respectively. The 5(6)-EET was significantly more potent than the other EETs ($P<0.0001$).

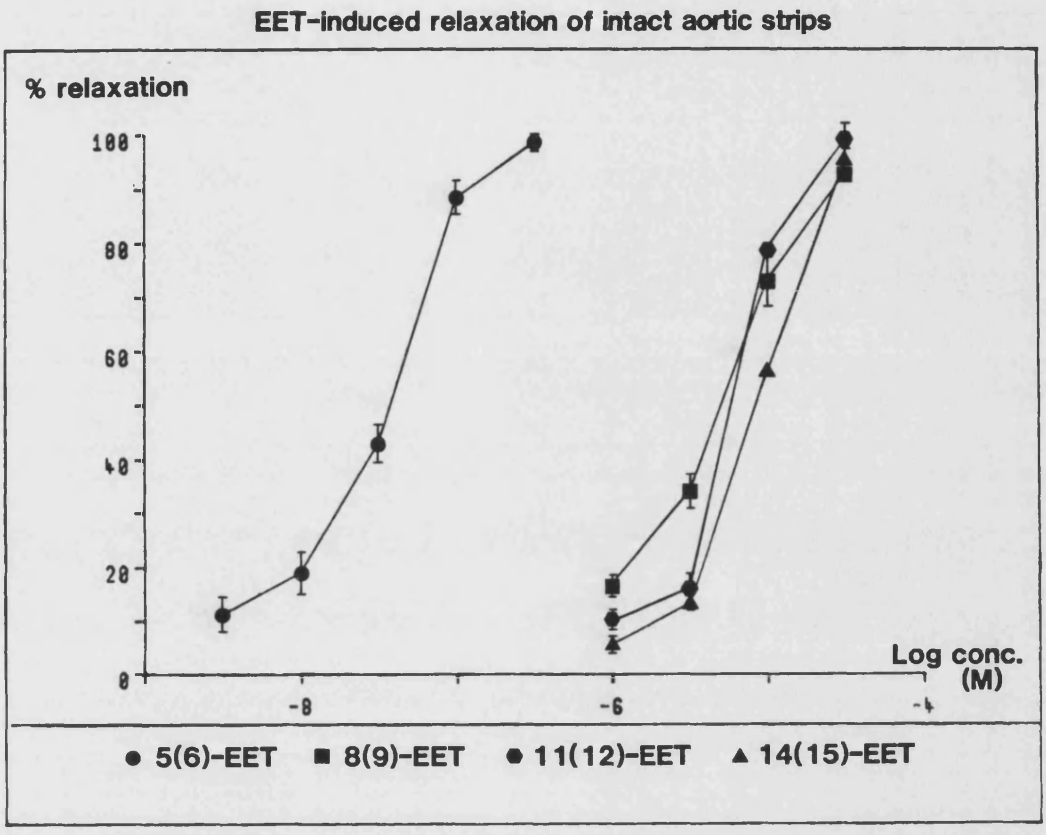


FIGURE 3.4

EET-induced relaxation of rat thoracic aorta with an intact endothelium. Cumulative addition of the EETs induced dose-dependent relaxations of rat aorta. 5(6)-EET induced relaxation with an EC_{50} value of $3.9 \pm 0.6 \times 10^{-8}M$ (n=4). 8(9)- 11(12)- and 14(15)-EET induced relaxation with EC_{50} values of $5.2 \pm 0.7 \times 10^{-8}M$ (n=4), $6.7 \pm 0.2 \times 10^{-6}M$ (n=3) and $8.6 \pm 0.2 \times 10^{-8}M$ (n=3) respectively.

3.4.3: Endothelial Cell Involvement in EET-induced Relaxation of Rat Aorta

Figure 3.5 shows a representative relaxant response to 5(6)-EET in (a) an intact aortic strip and (b) a denuded aortic strip.

In de-endothelialised strips (Fig. 3.6), the 5(6)-isomer was again the most potent, inducing relaxation in the range 10^{-8} to 10^{-6}M. The mean potency of 5(6)-EET in aortic strips with a disrupted endothelial cell lining was reduced in comparison to intact strips (EC_{50} value 8.2 $\pm$ 1.8 x 10^{-8}M (n=4) c.f 3.9 $\pm$ 0.6 x 10^{-8}M (n=4) in intact strips), however the change was not statistically significant (P = 0.08). The 8(9)-, 11(12)- and 14(15)-EETs were again relaxant in the range 10^{-6} to $3x10^{-5}$M and their EC_{50} values were comparable to those in intact preparations (8(9)-EET, 4.7 $\pm$ 1.7 x 10^{-6}M (n=3); 11(12)-EET, 6.5 $\pm$ 0.2 x 10^{-6}M (n=3); 14(15)-EET, 8.9 $\pm$ 0.8 x 10^{-6}M(n=3)). The 5(6)-EET was still significantly more potent than the other EETs ($P<0.001$). The potencies of 8(9)-, 11(12)- and 14(15)-EETs in de-endothelialised strips did not differ significantly from that in intact strips ($P>0.45$). Relaxant responses to the EETs are expressed as a percentage of maximal NaNP-induced relaxation.

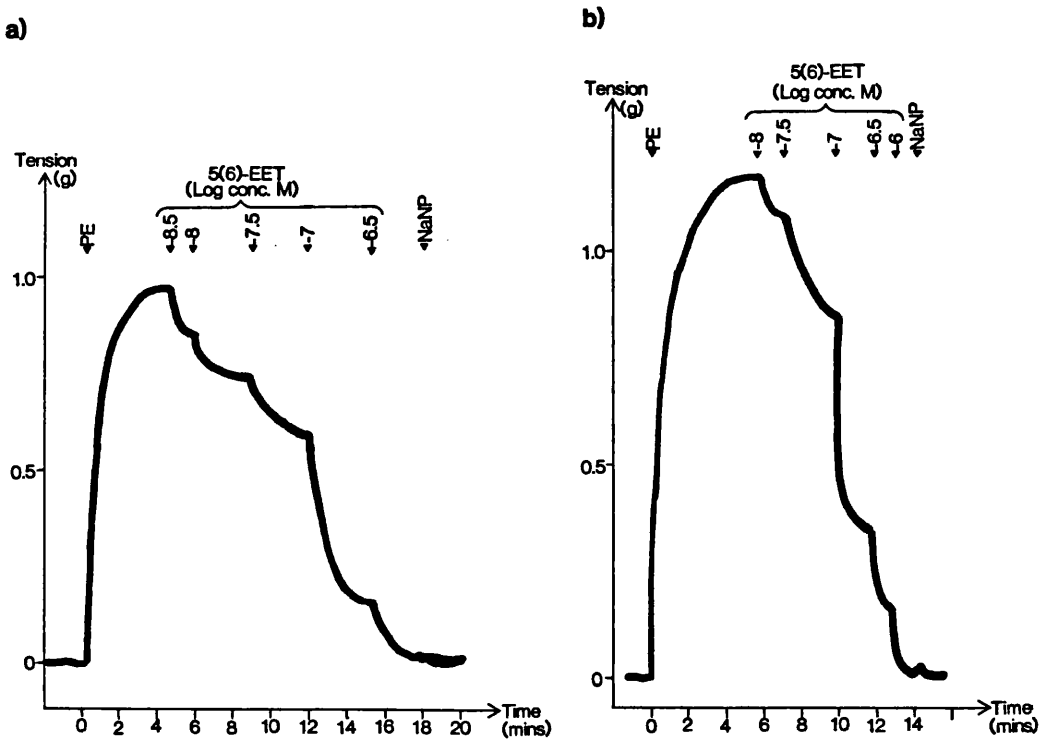


FIGURE 3.5
Respresentative traces of 5(6)-EET-induced relaxation of rat thoracic aorta a) with an intact endothelial lining and b) denuded of endothelium.

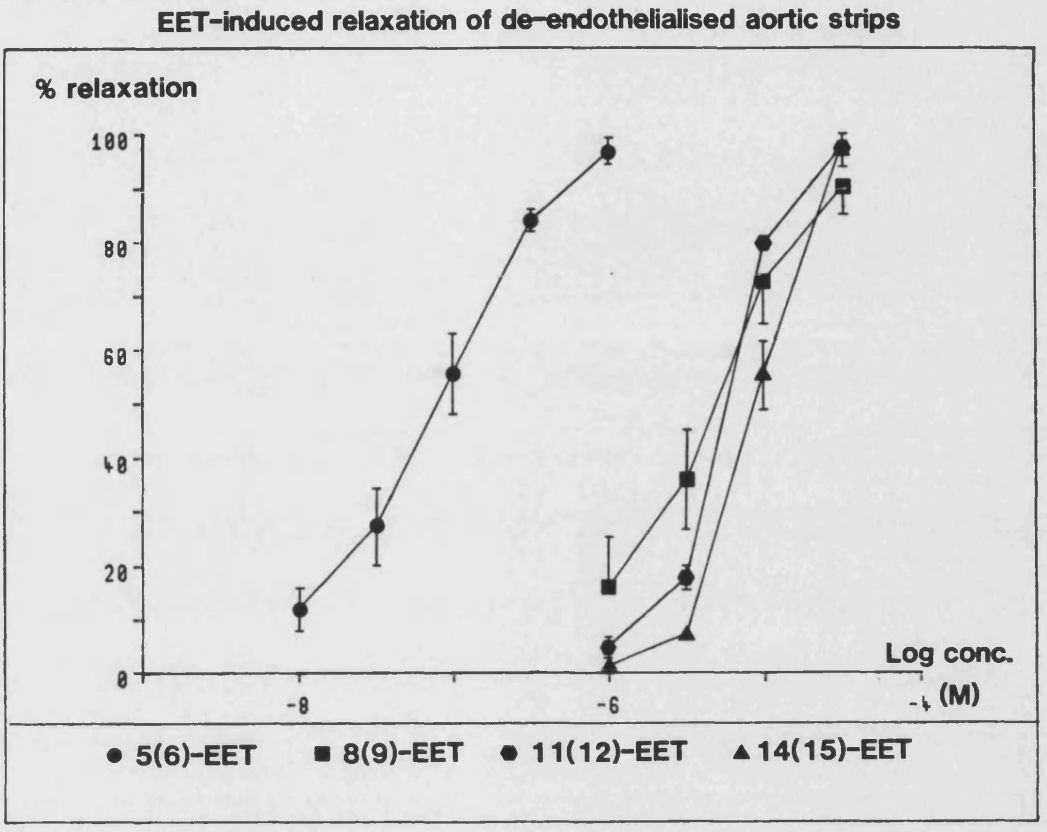


FIGURE 3.6

EET-induced relaxation of rat thoracic aorta denuded of an endothelial lining. Cumulative addition of the EETs induced a dose-dependent relaxation of rat aorta. The potency of the 5(6)-isomer was reduced by the removal of the endothelium whereas the potency of the other EETs was unaffected. 5(6)-EET induced relaxation with an EC_{50} value of 8.2 $\pm$ 1.8 x 10^{-8}M (n=4). 8(9)- 11(12)- and 14(15)-EET induced relaxations with EC_{50} values of 4.7 $\pm$ 1.7 x 10^{-6}M (n=3), 6.5 $\pm$ 0.2 x 10^{-6}M (n=3) and 8.9 $\pm$ 0.8 x 10^{-6}M (n=3) respectively.

3.4.4: Effects of Indomethacin and NDGA on EET-induced Relaxation of Rat Aorta

Exposure of aortic strips to $3x10^{-6}M$ indomethacin reduced the relaxant responses induced by 5(6)-EET (Fig. 3.7). 5(6)-EET-induced relaxation of intact aortic strips in the presence of indomethacin was observed between $10^{-8}M$ and $10^{-6}M$ with an EC_{50} of $7.5 \pm 1.0 \times 10^{-8}M$ (n=4), this compared to $3.9 \pm 0.6 \times 10^{-8}M$ in untreated strips demonstrating a significant reduction in potency ($P<0.02$). Pre-incubation with indomethacin had no significant ($P>0.45$) effect on 8(9)-, 11(12)- and 14(15)-EET induced relaxation. EC_{50} values for 8(9)-, 11(12)- and 14(15)-EET were $4.5 \pm 1.6 \times 10^{-6}M$ (n=4), $6.7 \pm 0.7 \times 10^{-6}M$ (n=4) and $8.5 \pm 0.8 \times 10^{-6}M$ (n=4) respectively. Neither the PE-induced contraction or the ACh-induced relaxation were altered in the presence of $3x10^{-6}M$ indomethacin.

5(6)-EET induced relaxations in de-endothelialised aortic strips was also attenuated by $3x10^{-6}M$ indomethacin. 5(6)-EET-induced relaxation of de-endothelialised strips pre-incubated with indomethacin was observed between $10^{-7}M$ and $10^{-5}M$ (EC_{50} value $1.2 \pm 0.3 \times 10^{-6}M$ (n=4)). Pre-incubation of de-endothelialised rat aortic strips with indomethacin again had no significant effect ($P>0.50$) on the relaxant responses observed with 8(9)-, 11(12)- and 14(15)-EET (EC_{50} values; $5.1 \pm 0.9 \times 10^{-6}M$ (n=4), $6.4 \pm 0.5 \times 10^{-6}M$ (n=4) and $8.1 \pm 1.1 \times$

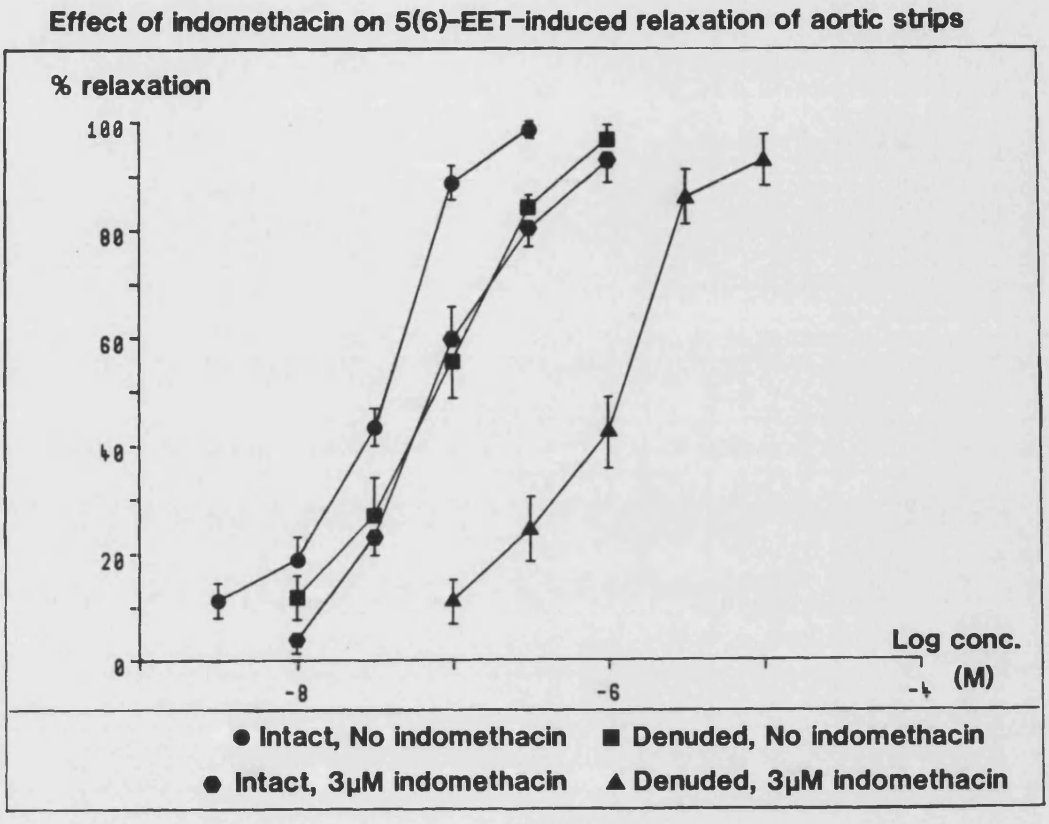


FIGURE 3.7

EET-induced relaxations of rat thoracic aortic strips in the presence and absence of indomethacin. The potency of 5(6)-EET in both intact and de-endothelialised aortic strips was reduced by pre-incubation of the aortic strips with 3×10^{-5}M indomethacin. EC_{50} values were $3.9 \pm 0.6 \times 10^{-8}$M (n=4) in intact untreated aorta, $7.5 \pm 1.0 \times 10^{-8}$M (n=4) in intact indomethacin treated aorta, $8.2 \pm 1.8 \times 10^{-8}$M (n=4) in de-endothelialised untreated aorta and $1.2 \pm 0.3 \times 10^{-5}$M (n=4) in de-endothelialised indomethacin treated aorta.

10^{-6}M (n=4) respectively).

Exposure of intact aortic strips to $1x10^{-6}$M NDGA had no significant effect (P>0.20) on EET-induced relaxations (Fig. 3.8). 5(6)-EET was again relaxant in the range $3x10^{-9}$M to $3x10^{-7}$M with an EC_{50} value of $3.1 \pm 0.7 \times 10^{-8}$M (n=4). The other three EETs were relaxant in the range $1x10^{-6}$M to $3x10^{-5}$M (EC_{50} values were $4.8 \pm 0.8 \times 10^{-6}$M for 8(9)-EET; $5.9 \pm 0.6 \times 10^{-6}$M for 11(12)-EET and $7.7 \pm 1.1 \times 10^{-6}$M for 14(15)-EET. All n=4).

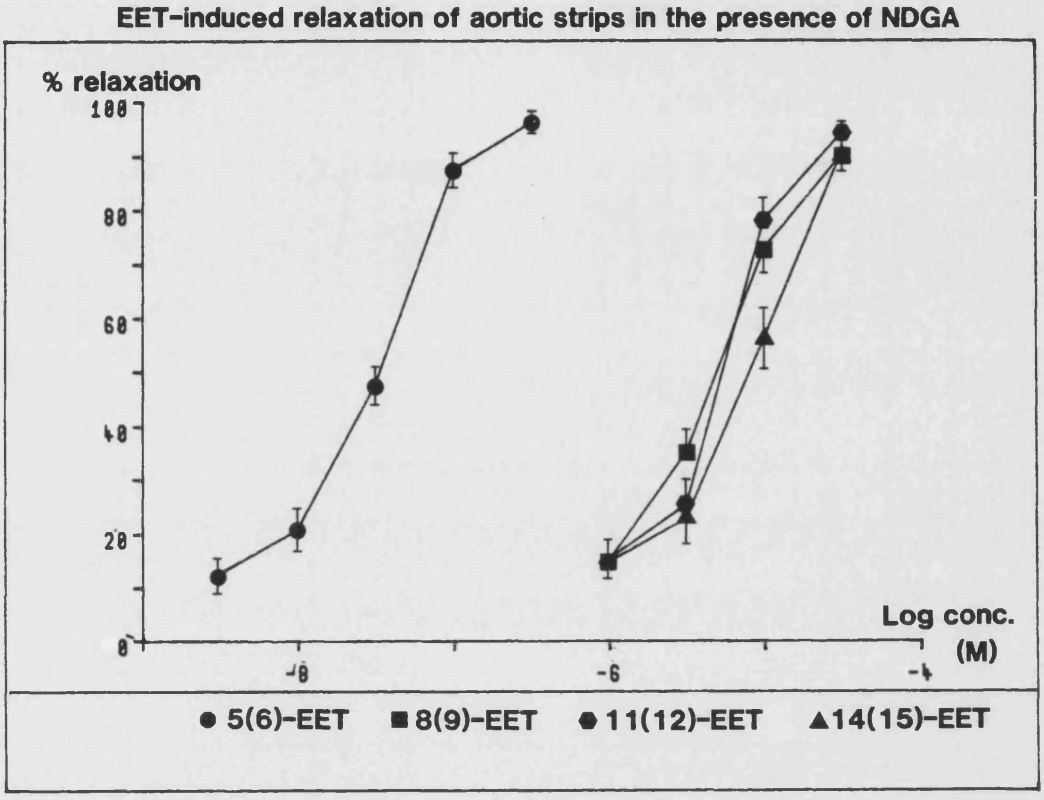


FIGURE 3.8

Effect of NDGA on EET-induced relaxation in rat aortic strips with an intact endothelial lining. Pre-incubation with 1×10^{-6}M NDGA had no significant effect on EET-induced relaxation. EC_{50} values in the presence of NDGA were $3.1 \pm 0.7 \times 10^{-8}$M (n=4), $4.8 \pm 0.8 \times 10^{-6}$M (n=4), $5.9 \pm 0.6 \times 10^{-6}$M (n=4) and $7.7 \pm 1.1 \times 10^{-6}$M (n=4) for 5(6)-, 8(9)-, 11(12)- and 14(15)-EET respectively.

3.4.5: Effect of M&B 22,948 on EET-induced Relaxations of Rat Aorta

Pre-incubation of intact rat aortic strips with $3x10^{-6}M$ M&B 22,948 for 5 minutes prior to the addition of PE significantly ($P<0.001$) attenuated the contractile response observed (Fig. 3.9). PE-induced contraction, in the presence of M&B 22,948 was observed in the range of $3x10^{-8}M$ to $3x10^{-6}M$ with an EC_{50} value of $1.7 \pm 0.1 \times 10^{-7}M$ ($n=6$) (c.f. EC_{50} of $9.8 \pm 0.7 \times 10^{-9}M$ ($n=6$) in the absence of M&B 22,948). ACh- and NaNP-induced relaxant responses were both augmented by pre-incubation with M&B 22,948. In the presence of M&B 22,948, ACh induced relaxation in the range $1x10^{-10}M$ to $1x10^{-7}M$ with an EC_{50} value of $3.1 \pm 0.7 \times 10^{-9}$ ($n=6$) (c.f. $1.7 \pm 0.3 \times 10^{-8}M$ ($n=6$) in the absence of M&B 22,948; $P<0.002$) (Fig. 3.10), and NaNP induced relaxation in the range $1x10^{-11}M$ to $3x10^{-8}M$ with an EC_{50} value of $7.0 \pm 0.8 \times 10^{-10}$ ($n=6$) (c.f. $2.0 \pm 0.2 \times 10^{-9}M$ ($n=6$) in the absence of M&B 22,948; $P<0.001$) (Fig. 3.11).

Due to the relaxatory effect of M&B 22,948, exerted through its augmentation of EDRF in intact aortic strips, subsequent investigations utilised de-endothelialised strips. Relaxations of de-endothelialised strips induced by 8(9)-, 11(12)- and 14(15)-EET were significantly ($P<0.002$) augmented in the presence of M&B 22,948 (Fig. 3.12). The 5(6)-EET-induced response was unaltered ($P>0.90$), relaxation being observed in the range $3x10^{-9}M$

to 1×10^{-6}M with an EC_{50} value of $7.8 \pm 1.8 \times 10^{-8}$M (n=4) (c.f. $8.5 \pm 1.6 \times 10^{-8}$M (n=4) in the absence of M&B 22,948). 8(9)-, 11(12)- and 14(15)-EETs induced relaxation in the range 10^{-7}M to 10^{-5}M with EC_{50} values of $1.2 \pm 0.3 \times 10^{-6}$M (n=4), $1.8 \pm 0.2 \times 10^{-6}$M (n=4) and $1.6 \pm 0.2 \times 10^{-6}$M (n=4) respectively (c.f. $5.9 \pm 0.9 \times 10^{-6}$M (n=4), $6.5 \pm 0.7 \times 10^{-6}$M (n=4) and $7.2 \pm 1.0 \times 10^{-6}$M (n=4) respectively in the absence of M&B 22,948).

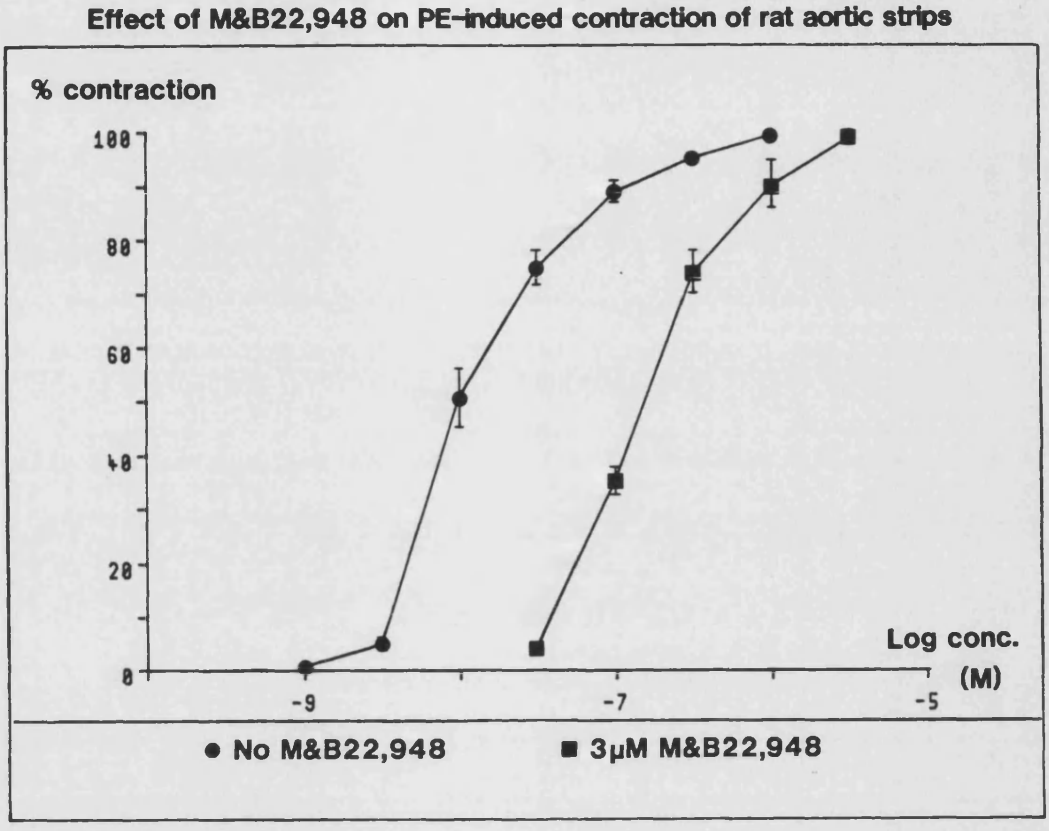


FIGURE 3.9

Effect of M&B 22,948 on PE-induced contraction of rat thoracic aortic strips with an intact endothelial lining. The potency of PE was decreased following pre-incubation of the tissues with M&B 22,948. In the absence of M&B 22,948, PE induced contraction with an EC_{50} of 9.8 $\pm$ 0.7 x 10^{-9}M (n=6). In the presence of M&B 22,948 the EC_{50} for PE-induced contraction increased to 1.7 $\pm$ 0.1 x 10^{-7}M (n=6).

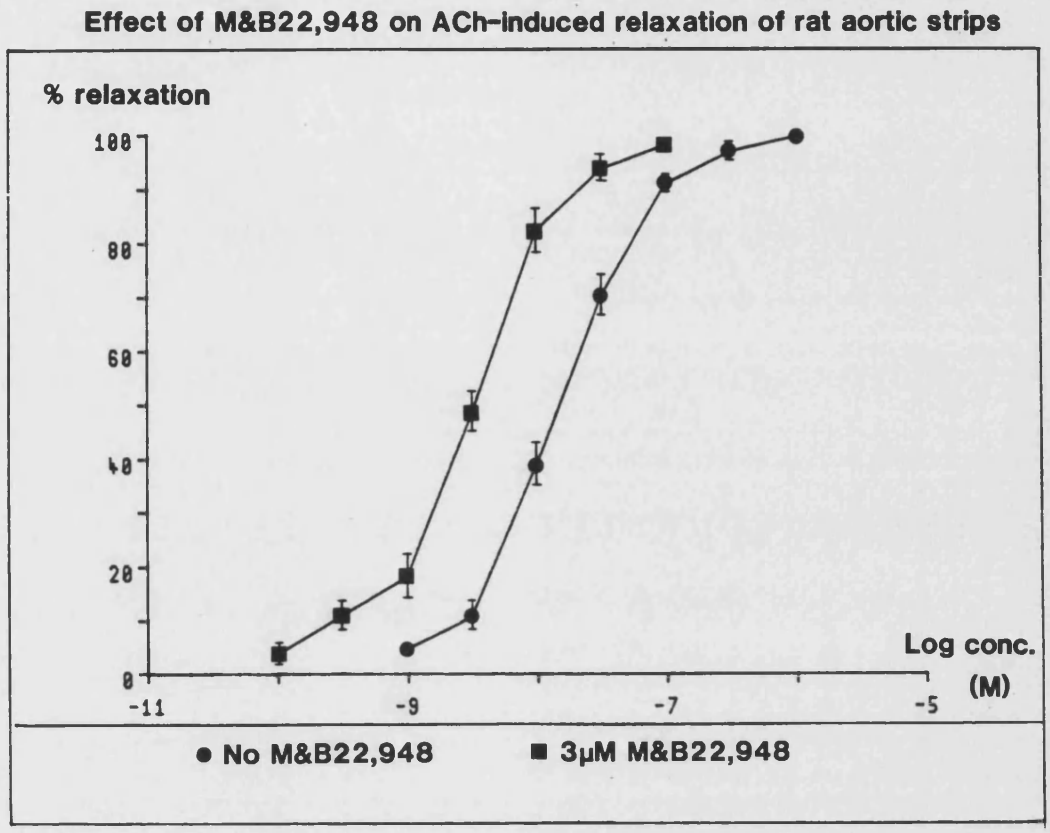


FIGURE 3.10

Effect of M&B 22,948 on ACh-induced relaxation of rat thoracic aortic strips with an intact endothelial lining. The potency of ACh was increased following pre-incubation of the tissues with M&B 22,948. In the absence of M&B 22,948, ACh induced relaxation with an EC_{50} of 1.7 $\pm$ 0.3 x 10^{-8}M (n=6). In the presence of M&B 22,948 the EC_{50} for ACh-induced relaxation decreased to 3.1 $\pm$ 0.7 x 10^{-9}M (n=6).

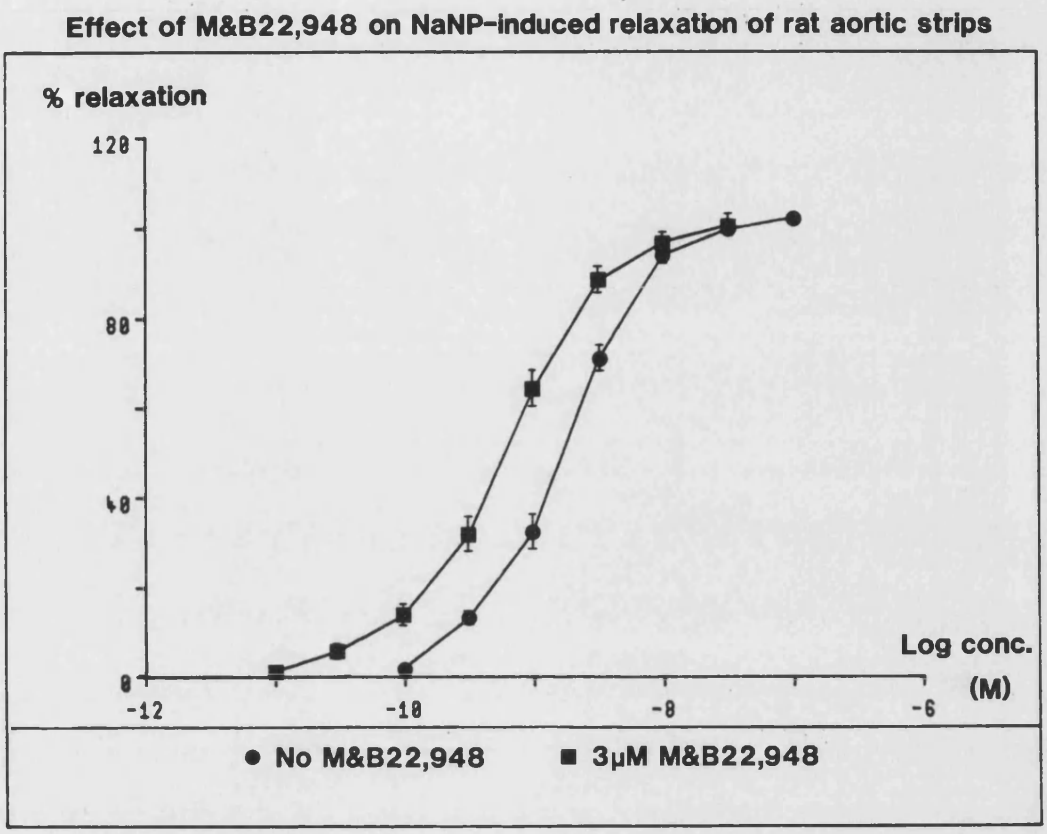


FIGURE 3.11

Effect of M&B 22,948 on NaNP-induced relaxation of rat thoracic aortic strips with an intact endothelial lining. The potency of NaNP was increased following pre-incubation of the tissues with M&B 22,948. In the absence of M&B 22,948, NaNP induced relaxation with an EC_{50} of 2.0 $\pm$ 0.2 x 10^{-9}M (n=6). In the presence of M&B 22,948 the EC_{50} for NaNP-induced relaxation decreased to 7.0 $\pm$ 0.8 x 10^{-10}M (n=6).

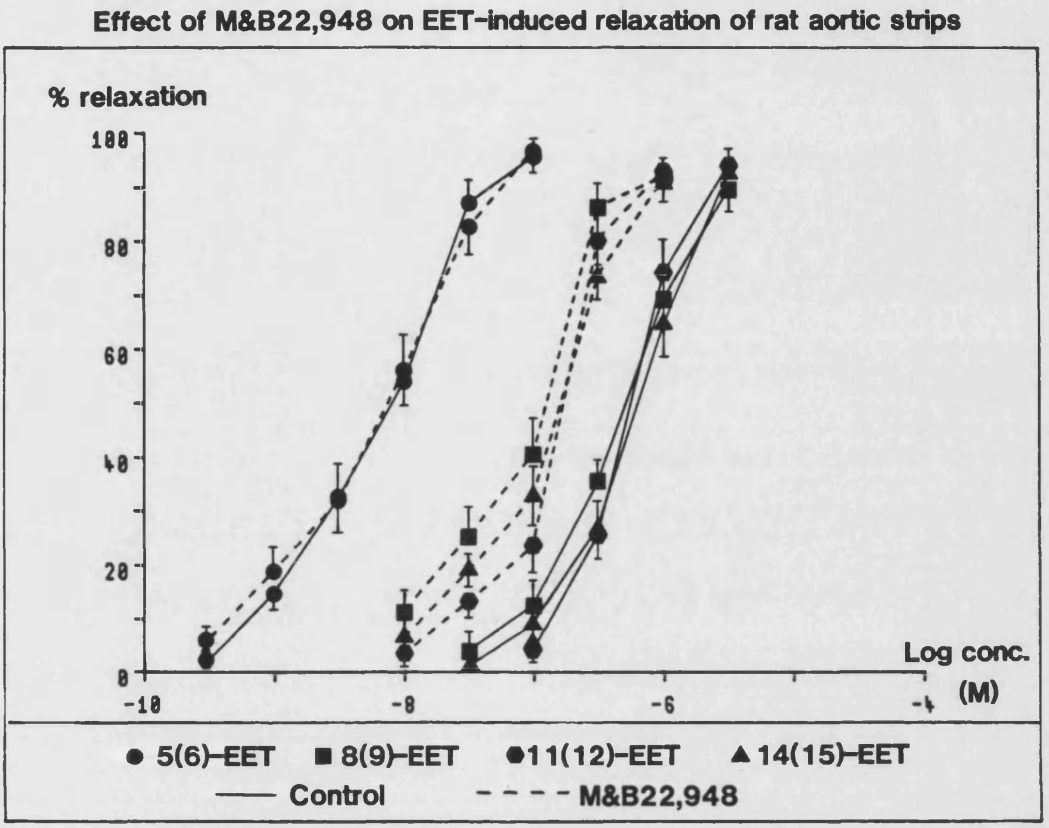


FIGURE 3.12

Effect of M&B 22,948 on EET-induced relaxation of rat thoracic aortic strips denuded of endothelium. The potencies of 8(9)-, 11(12)- and 14(15)-EETs were increased following pre-incubation of the tissues with M&B 22,948, however the response to 5(6)-EET was unaltered. The EC_{50} values for the individual EETs in the presence of M&B 22,948 (c.f. absence of M&B 22,948) were: for 5(6)-EET 7.8 $\pm$ 1.8 x 10^{-8}M (c.f. 8.5 $\pm$ 1.6 x 10^{-8}M); for 8(9)-EET 1.2 $\pm$ 0.3 x 10^{-6}M (c.f. 5.9 $\pm$ 0.9 x 10^{-6}M); for 11(12)-EET 1.8 $\pm$ 0.2 x 10^{-6}M (c.f. 6.5 $\pm$ 0.7 x 10^{-6}M); and for 14(15)-EET 1.6 $\pm$ 0.2 x 10^{-6}M (c.f. 7.2 $\pm$ 1.0 x 10^{-6}M). n=4.

3.5: RESULTS: GUINEA-PIG TRACHEA

3.5.1: Control Responses

After two hours a stable basal tone of approximately 1 to 1.5g was reached. Tracheal strips exposed to indomethacin throughout the equilibration period continually decreased in tone finally aquiring a resting tension of approximately 1g (Fig. 3.13a). However tracheal strips not exposed to indomethacin initially demonstrated a reduction in tone for approximately 40 minutes, followed by an increase in tone to a final resting tension of approximately 1.5g (Fig. 3.13b).

Drug induced tension was developed in the tissue using ACh concentrations greater than 10^{-7}M (Fig. 3.14). A maximal response to ACh was not achieved as exposure to ACh concentrations greater than 10^{-3}M rendered the tissue less responsive to subsequent drug applications. A suitable sustainable submaximal tone of 2g was induced by $3x10^{-6}$M and $1x10^{-5}$M ACh in the presence and absence of indomethacin respectively. The increase in sensitivity of the tracheal strips to ACh-induced tone was significant at the higher concentrations ($P<0.05$). NaNP-induced relaxations were obtained in the range 10^{-7}M to 10^{-3}M, with EC_{50} values of $2.0 \pm 0.5 \times 10^{-6}$M (n=7) and $3.0 \pm 0.6 \times 10^{-6}$M (n=8) for control and indomethacin treated tissues respectively (Fig. 3.15). In the presence of 3uM

indomethacin the maximal NaNP-induced relaxation was significantly less than in tissues not treated with indomethacin ($P<0.03$). Maximal relaxation with no indomethacin was $100.4 \pm 10.3\%$ as opposed to $75.6 \pm 2.4\%$ in the presence of indomethacin.

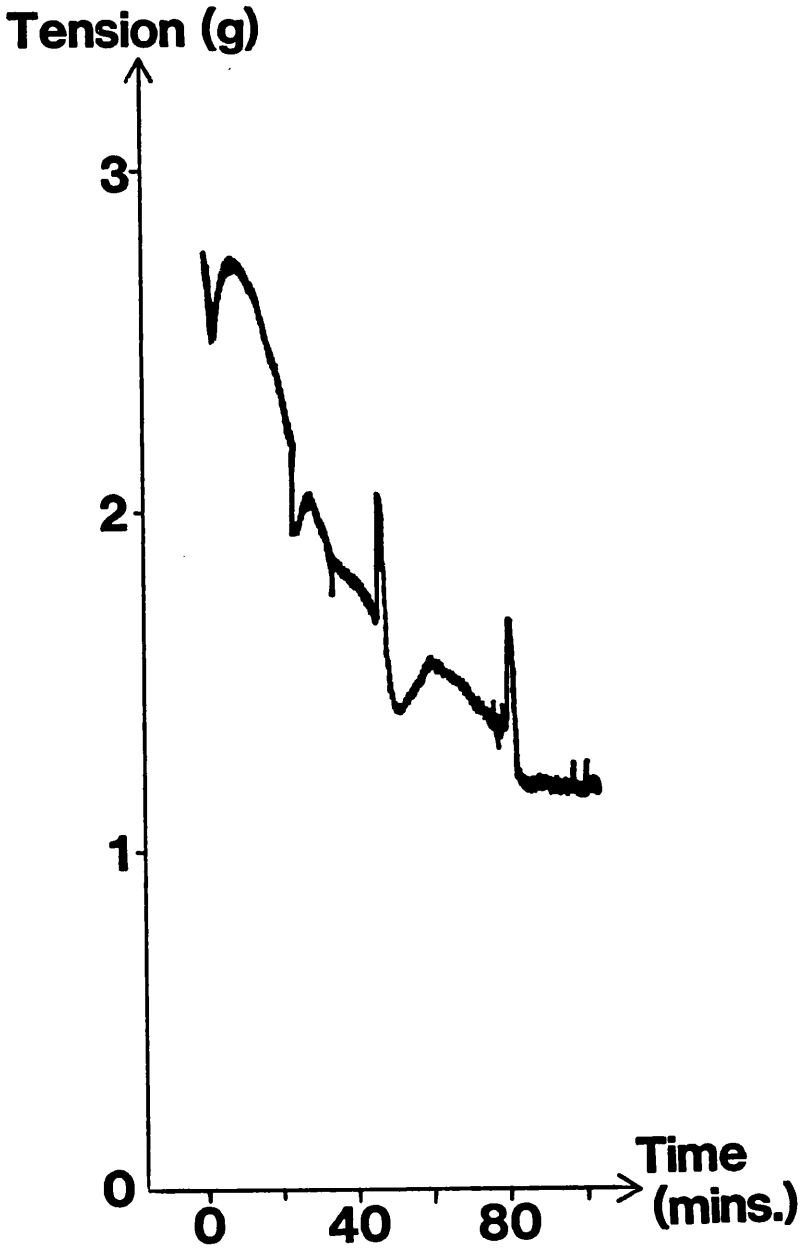


FIGURE 3.13a
Representative trace showing the continual decrease in basal tone of guinea-pig isolated tracheal rings throughout equilibration in the presence of indomethacin. The final basal tone of tracheal rings incubated with indomethacin was always less than that of untreated rings.

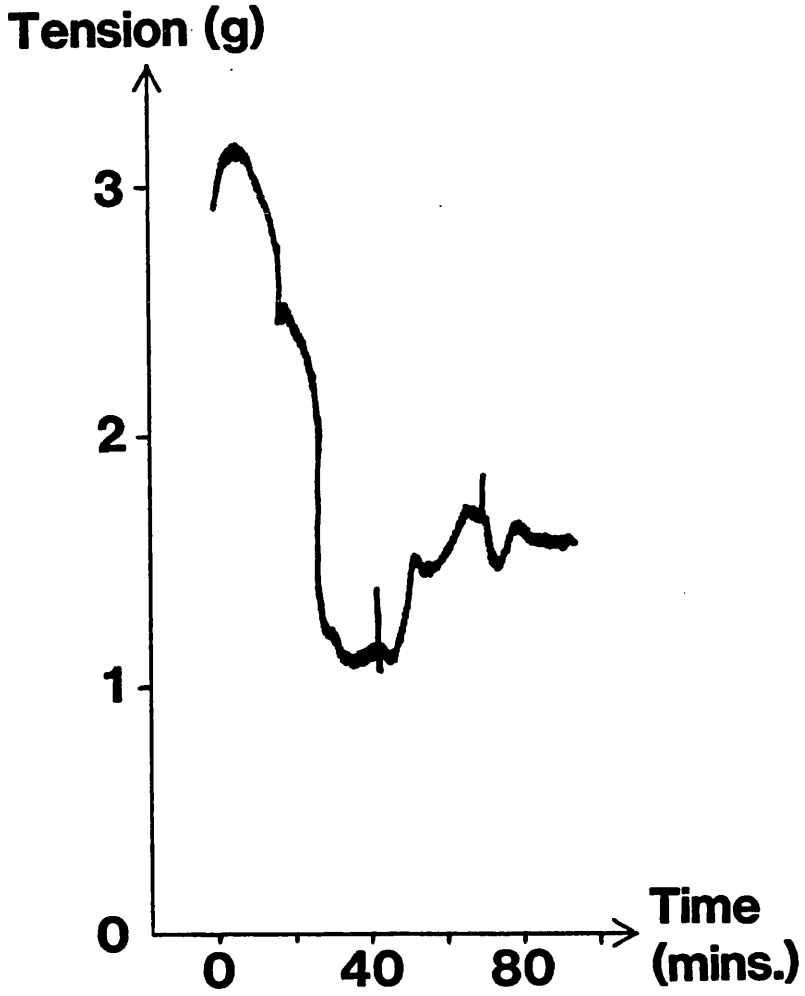


FIGURE 3.13b
Representative trace showing an increase in basal tone during equilibration of guinea-pig isolated tracheal rings in the absence of indomethacin. This increase characteristically occured after 40 minutes.

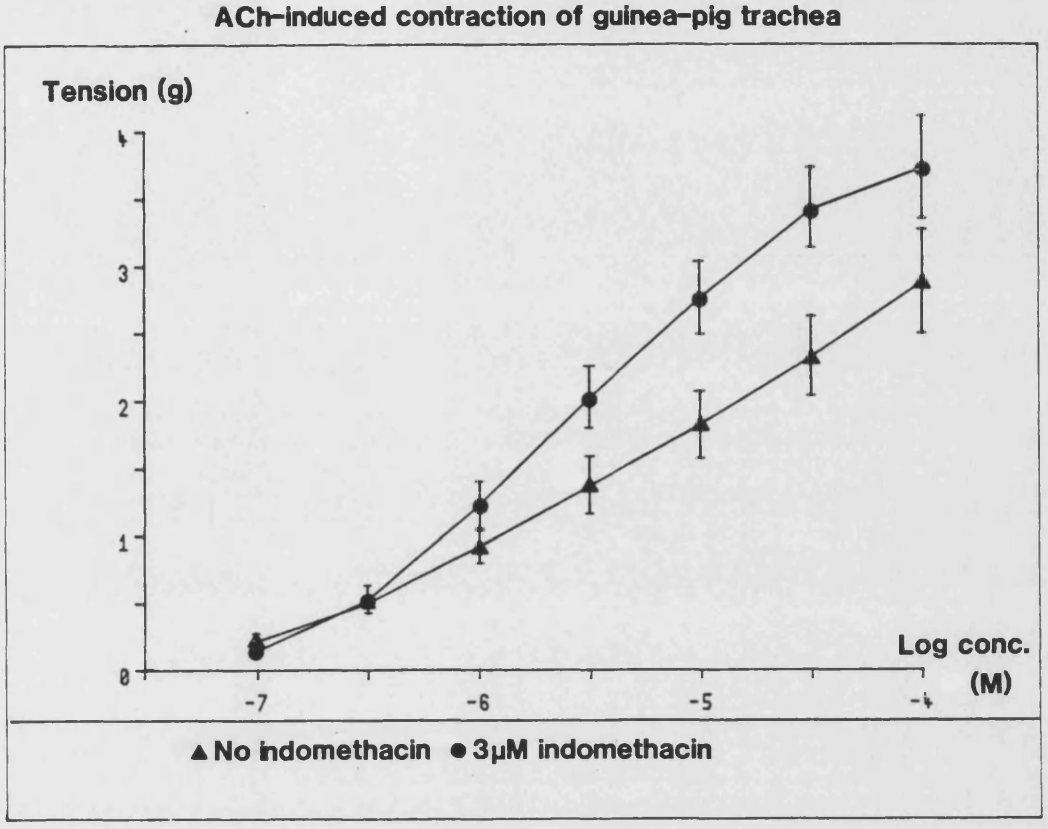


FIGURE 3.14

ACh-induced contraction of guinea-pig tracheal rings in the presence and absence of $3x10^{-6}$M indomethacin. Exposure of the tracheal rings to indomethacin increase the magnitude of response observed to ACh. EC_{50} values for ACh-induced contractions were not calculated as maximal responses could not be obtained without causing lasting desensitization to the preparations.

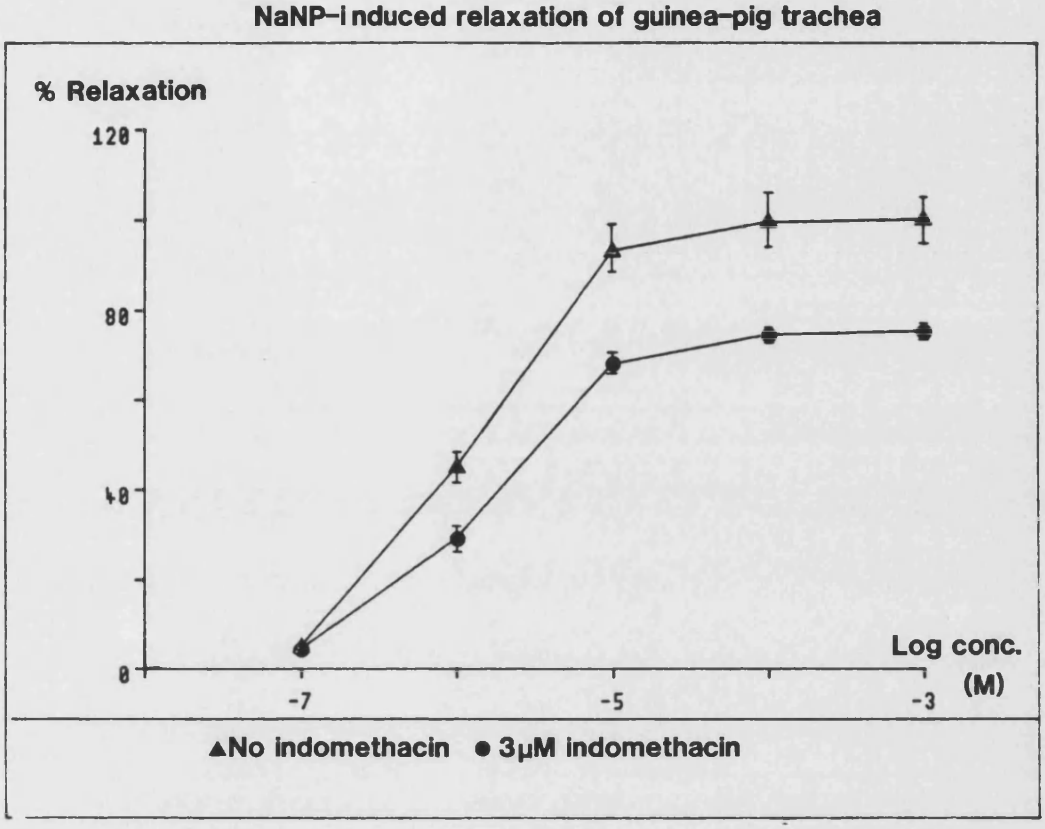


FIGURE 3.15

NaNP-induced relaxation of guinea-pig tracheal rings in the presence and absence of 3×10^{-6}M indomethacin. Exposure of the tracheal rings to indomethacin decrease the magnitude of response observed to NaNP. EC_{50} values for NaNP-induced relaxation were $2.0 \pm 0.5 \times 10^{-6}$M (n=7) in the absence of indomethacin and $3.0 \pm 0.6 \times 10^{-6}$M (n=8) in the presence of indomethacin.

3.5.2: EET-induced Relaxation of Guinea-pig Trachea

All four EETs induced a dose-dependent relaxation in guinea-pig tracheal strips (Fig. 3.16). 5(6)-EET induced a dose-dependent relaxation in the range 10^{-7}M to $3x10^{-5}$M with an EC_{50} value of 1.3 $\pm$ 0.5 x 10^{-4}M (n=8). The 5(6)-EET was significantly more potent then the other three EETs ($P<0.01$). 8(9)-, 11(12)- and 14(15)-EET were roughly equipotent and induced relaxation in the range 10^{-5}M to $3x10^{-4}$M. The EC_{50} values for 8(9)-, 11(12)- and 14(15)-EET were 7.3 $\pm$ 0.4 x 10^{-5}M (n=5), 2.8 $\pm$ 1.6 x 10^{-5}M (n=5) and 6.4 $\pm$ 0.3 x 10^{-5}M (n=4) respectively. Maximal relaxation observed with $3x10^{-5}$M 5(6)-EET was only 60.6% $\pm$ 5.0% of maximal NaNP-induced relaxation. Maximal relaxation observed with the other three isomers at a concentration of $3x10^{-4}$M to $1x10^{-3}$M was approximately 80-100% of maximal NaNP-induced relaxation.

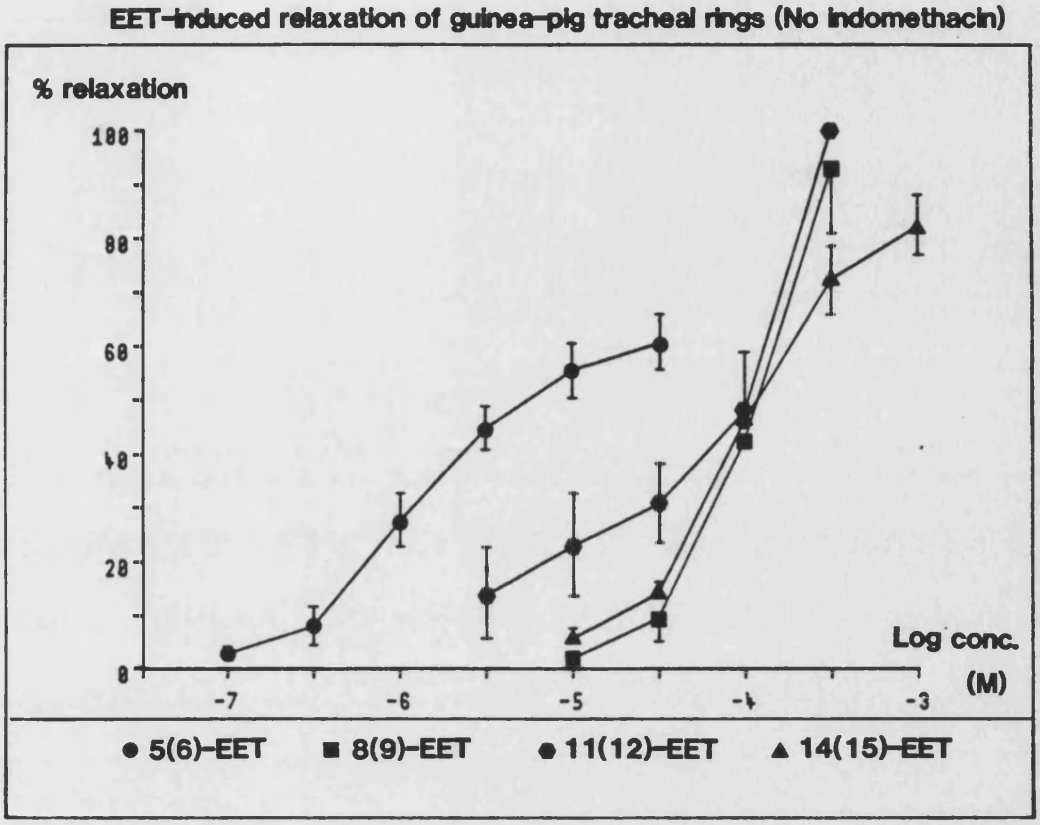


FIGURE 3.16

EET-induced relaxation of guinea-pig tracheal rings. All four EET-isomers induced a dose dependent relaxation of trachea. 5(6)-EET was more potent than the other isomers and induced relaxation with an EC_{50} of 1.3 $\pm$ 0.5 x 10^{-6}M (n=8). EC_{50} values for the 8(9)-, 11(12)- and 14(15)-EETs were 7.3 $\pm$ 0.4 x 10^{-5}M (n=5), 2.8 $\pm$ 1.6 x 10^{-5}M (n=5) and 6.4 $\pm$ 0.3 x 10^{-5}M (n=4) respectively.

3.5.3: Effects of Indomethacin and NDGA on EET-induced Relaxation of Guinea-pig Trachea

Figure 3.17 shows a representative relaxation response of a guinea-pig tracheal strip to 5(6)-EET in (a) the absence of indomethacin and (b) the presence of indomethacin.

Exposure of the trachea to 3×10^{-6}M indomethacin reduced the potency of 5(6)-EET (Fig. 3.18). In the presence of indomethacin 5(6)-EET induced relaxation in the range 1×10^{-6}M to 3×10^{-4}M with an EC_{50} value of $4.3 \pm 0.6 \times 10^{-5}$M (n=4). 8(9)-, 11(12)- and 14(15)-EET induced relaxation in the range 1×10^{-5}M to 3×10^{-4}M and had EC_{50} values of $1.4 \pm 0.3 \times 10^{-4}$M (n=3), $1.2 \pm 0.3 \times 10^{-4}$M (n=3) and $9.9 \pm 1.8 \times 10^{-5}$M (n=3) respectively (Fig. 3.19). 8(9)-, 11(12)- and 14(15)-EET induced relaxations in the presence of indomethacin were not significantly different from that in the absence of indomethacin ($P>0.30$). In the presence of indomethacin the maximal 5(6)-EET induced relaxation (76.3% $\pm$ 4.0%) was comparable to that induced by the other three EETs.

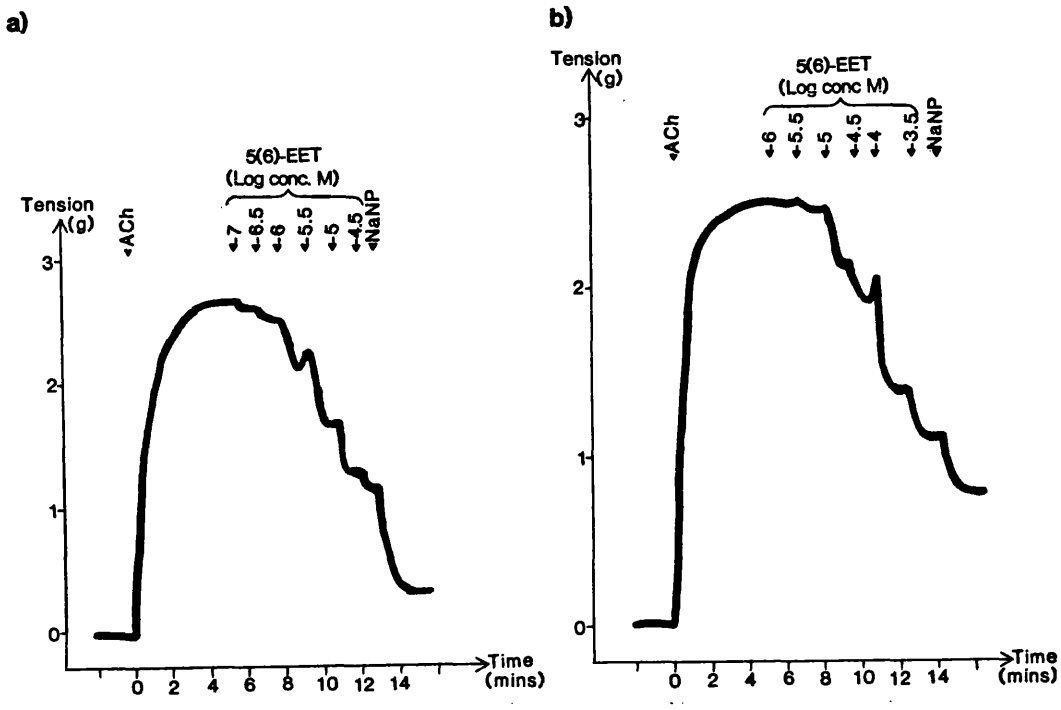


FIGURE 3.17
Representative traces of 5(6)-EET-induced relaxation of guinea-pig isolated tracheal rings in the a) absence of indomethacin and b) presence of indomethacin.

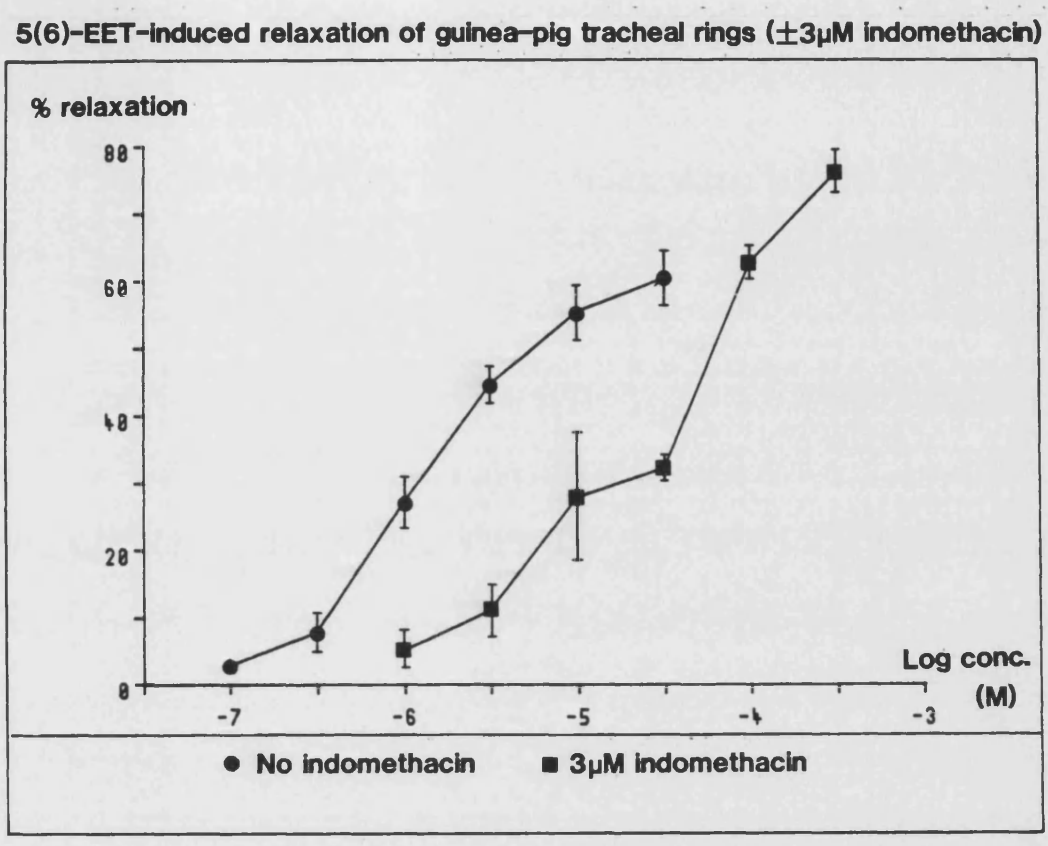


FIGURE 3.18

5(6)-EET-induced relaxation in the presence and absence of indomethacin. Direct comparison of the two dose-response curves highlights the reduced potency but increased efficacy of the 5(6)-isomer following exposure of tracheal to indomethacin.

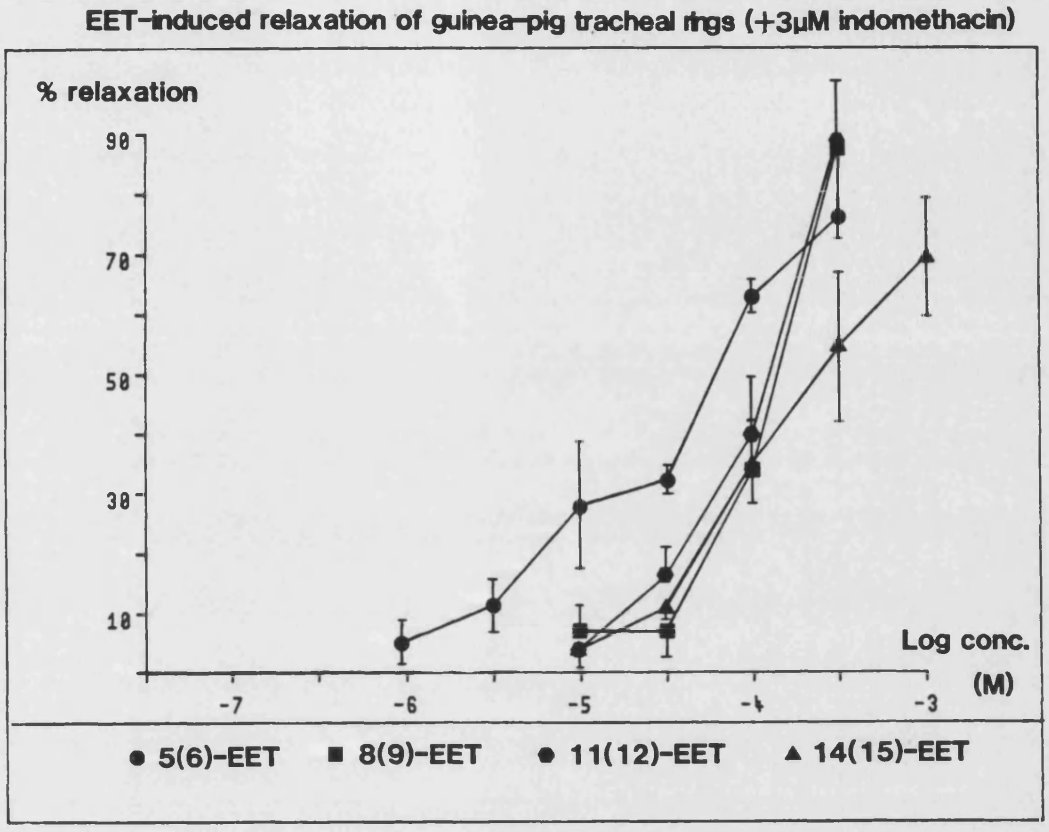


FIGURE 3.19

EET-induced relaxation of guinea-pig tracheal strips in the presence of 3×10^{-6}M indomethacin. All four EET-isomers induced a dose dependent relaxation of trachea. The potency of 5(6)-EET was reduced in the presence of indomethacin, $EC_{50} = 4.3 \pm 0.6 \times 10^{-5}$M (n=4). The responses to the other EETs were unaltered by indomethacin, EC_{50} values for the 8(9)-, 11(12)- and 14(15)-EETs were $1.4 \pm 0.3 \times 10^{-4}$M (n=3), $1.2 \pm 0.3 \times 10^{-4}$M (n=3) and $9.9 \pm 1.8 \times 10^{-5}$M (n=3) respectively. The degree of relaxation to 8(9)-, 11(12)- and 14(15)-EET was reduced in the presence of indomethacin.

Exposure of guinea-pig tracheal strips to $1x10^{-4}$M NDGA had no significant effect ($P>0.20$) on EET-induced relaxations (Fig. 3.20). 5(6)-EET induced relaxation in the range $1x10^{-7}$M to $3x10^{-5}$M with an EC_{50} value of $9.7 \pm 1.1 \times 10^{-7}$M (n=4). The other three EETs were relaxant in the range $1x10^{-5}$M to $1x10^{-3}$M (EC_{50} values were $1.0 \pm 0.1 \times 10^{-4}$M for 8(9)-EET, $9.5 \pm 1.6 \times 10^{-5}$M for 11(12)-EET and $9.5 \pm 1.5 \times 10^{-5}$M for 14(15)-EET. All n=4).

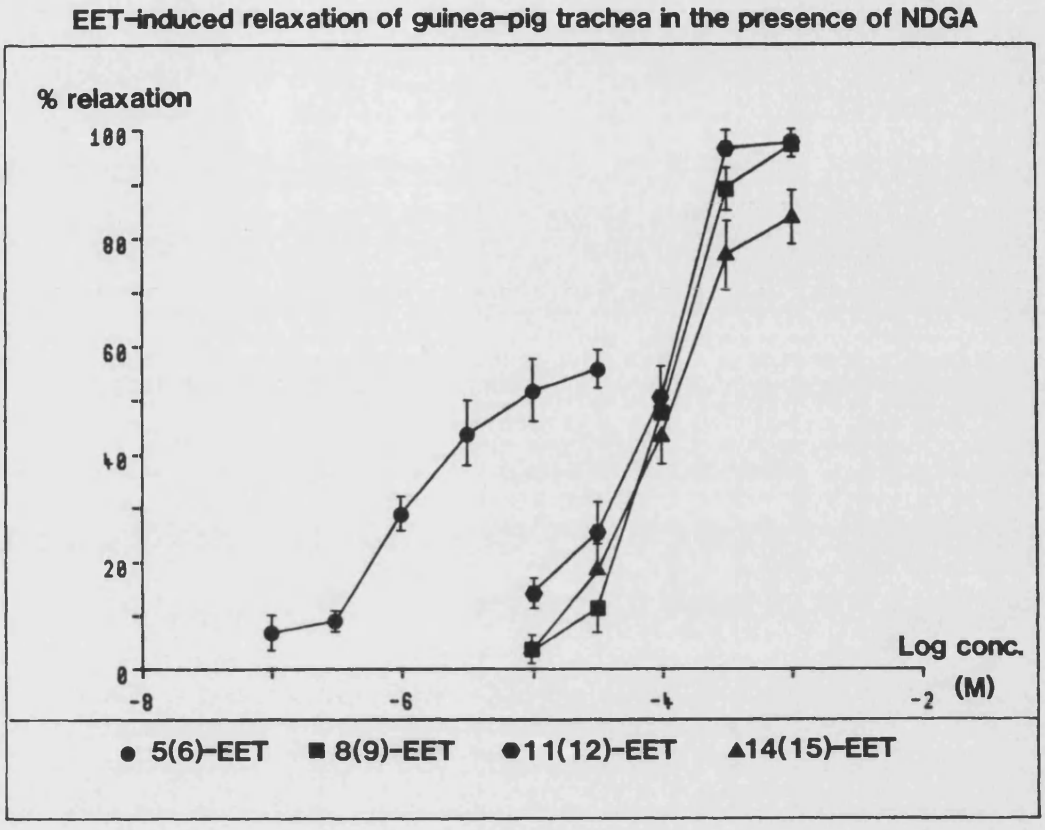


FIGURE 3.20

Effect of NDGA on EET-induced relaxation in guinea-pig tracheal rings. Pre-incubation with 1×10^{-6}M NDGA had no significant effect on EET-induced relaxation. EC_{50} values in the presence of NDGA were $9.7 \pm 1.1 \times 10^{-7}$M (n=4), $1.0 \pm 0.1 \times 10^{-4}$M (n=4), $9.5 \pm 1.6 \times 10^{-5}$M (n=4) and $9.5 \pm 1.5 \times 10^{-5}$M (n=4) for 5(6)-, 8(9)-, 11(12)- and 14(15)-EET respectively.

3.5.4: Effect of M&B 22,948 on EET-induced Relaxation of Guinea-pig Trachea

Pre-incubation of guinea-pig tracheal strips with $3x10^{-6}$M M&B 22,948 for 5 minutes prior to the addition of ACh had no effect on ACh-induced contractions. Although NaNP-induced relaxations were augmented by M&B 22,948 the increase in potency was not significant (P=0.06), EC_{50} value for NaNP-induced relaxation in the presence of $3x10^{-6}$M M&B 22,948 was 4.7 $\pm$ 0.8 x 10^{-7}M (n=4) (c.f. 1.3 $\pm$ 0.4 x 10^{-6}M (n=4) in the absence of M&B 22,948) (Fig. 3.21). 8(9)-, 11(12)- and 14(15)-EET induced relaxations of guinea-pig tracheal strips were significantly ($P<0.002$) augmented in the presence of M&B 22,948, however 5(6)-EET-induced relaxation was not significantly ($P>0.20$) altered (Fig. 3.22). 5(6)-EET-induced relaxation in the range $1x10^{-7}$M to $3x10^{-5}$M with an EC_{50} of 2.0 $\pm$ 0.4 x 10^{-6}M (n=4) (c.f. 1.4 $\pm$ 0.6 x 10^{-6}M (n=4) in the absence of M&B 22,948). 8(9)-, 11(12)- and 14(15)-EETs induced relaxation in the range $1x10^{-6}$M to $3x10^{-4}$M with EC_{50} values of 4.9 $\pm$ 0.7 x 10^{-5}M (n=4), 3.5 $\pm$ 0.8 x 10^{-5}M (n=4) and 3.7 $\pm$ 1.2 x 10^{-5}M (n=4) respectively (c.f. 1.3 $\pm$ 0.2 x 10^{-4}M (n=4) for 8(9)-EET; 1.2 $\pm$ 0.2 x 10^{-4}M (n=4) for 11(12)-EET and 9.2 $\pm$ 0.9 x 10^{-5}M (n=4) for 14(15)-EET).

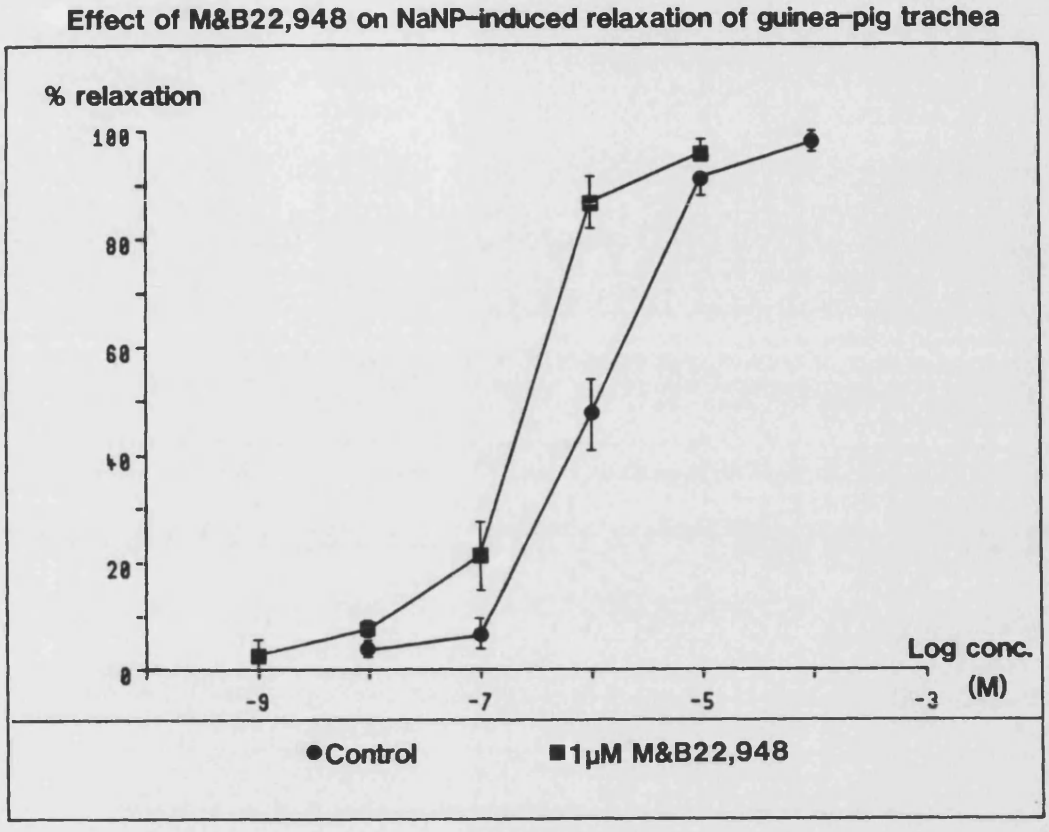


FIGURE 3.21

Effect of M&B 22,948 on NaNP-induced relaxation of guinea-pig tracheal rings. The potency of NaNP was increased following pre-incubation of the tissues with M&B 22,948. In the absence of M&B 22,948, NaNP induced relaxation with an EC_{50} of 1.3 $\pm$ 0.4 x 10^{-8}M (n=4). In the presence of M&B 22,948 the EC_{50} for NaNP-induced relaxation decreased to 4.7 $\pm$ 0.8 x 10^{-7}M (n=4).

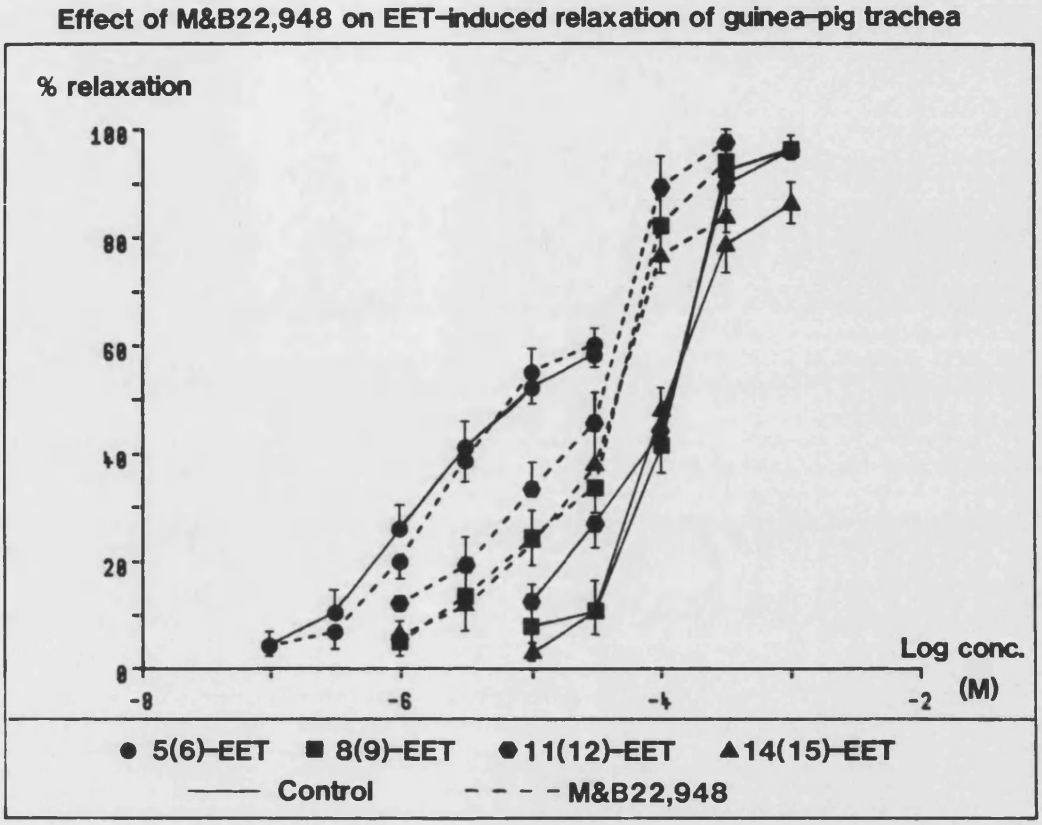


FIGURE 3.22

Effect of M&B 22,948 on EET-induced relaxation of guinea-pig tracheal rings. The potencies of the 8(9)-, 11(12)- and 14(15)- EET-isomers was increased following pre-incubation of the tissues with M&B 22,948. The response to 5(6)-EET was unaltered. The EC_{50} values for the individual EETs in the presence of M&B 22,948 (c.f. absence of M&B 22,948) were: for 5(6)-EET 2.0 $\pm$ 0.4 x 10^{-6}M (c.f. 1.4 $\pm$ 0.6 x 10^{-6}M); for 8(9)-EET 4.9 $\pm$ 0.7 x 10^{-5}M (c.f. 1.3 $\pm$ 0.2 x 10^{-4}M); for 11(12)-EET 3.5 $\pm$ 0.8 x 10^{-5}M (c.f. 1.2 $\pm$ 0.3 x 10^{-4}M); and for 14(15)-EET 3.7 $\pm$ 1.2 x 10^{-5}M (c.f. 9.2 $\pm$ 0.9 x 10^{-5}M). n=4.

3.6: DISCUSSION

3.6.1a: Aortic Responses

The observation that aortic strips with an intact endothelial lining, taken from rats with a body weight of less than 300-330g, were not capable of maintaining a stable PE-induced contracture was not further investigated. Some indication of the possible cause was demonstrated by the ability of de-endothelialised strips to maintain a steady contraction to PE. This would suggest the participation of the endothelium and possibly EDRF. A similar observation was reported by Ignarro et al., (1987). Small branches of unrubbed bovine intrapulmonary arteries and veins were less able to maintain a steady level of sub-maximal PE-induced contraction than larger branches. Ignarro et al. (1987) suggested that the larger endothelium:smooth muscle ratio found in smaller vessels could be responsible. Alternatively, in this case, the greater age of the larger rats must also be considered. The ability of the endothelial lining to release EDRF may be impaired with age.

Consistent with the theory of a basal release of EDRF (Griffith et al. 1984; Martin et al., 1985) is the difference in responses to PE observed in intact and de-endothelialised aortic strips. These differences were evident in strips taken from the same area of the same

aorta. Such a basal release is also suggested by the reduced sensitivity of intact aortic strips to PE in the presence of M&B 22,948 (see later).

3.6.1b: Tracheal Responses

The differences in basal tone, ACh-induced contraction and NaNP-induced relaxation between tracheal rings in the presence and absence of indomethacin are probably a result of spontaneously-released eicosanoids. The involvement of eicosanoids in indomethacin potentiation of agonist-induced contractions in guinea-pig tracheal preparations has been described by Orehek et al., (1975). The reduction in basal tone was suggested to be a result of decreased $PGF_{2\alpha}$ synthesis (Orehek et al. (1975), such a basal release being supported by the observations of Tschirhart et al., (1987). Furthermore, attenuation of ACh-induced contractions at low doses by indomethacin was also observed. Alternatively, basal tone may result from the action of PGE_2 on EP_1 receptors (Dond et al., 1986) stimulating the IP_3/DAG pathway. The eicosanoid responsible for opposing agonist-induced contraction has been identified as PGE_2 (Orehek et al., 1975), in this case PGE_2 would exert its effects through the stimulation of the EP_2 receptor. Furthermore Orehek et al. (1975) demonstrated that these alterations in contractile and relaxant responses were not simply a consequence of differences in initial resting tension. Whilst the

potentiation of histamine-induced contractions of guinea-pig tracheal strips by indomethacin or de-epithelialisation is readily acknowledged, such potentiations of ACh-induced contractions have also been reported (Flavahan, 1985; Holryod, 1986; Murlas, 1986).

Lipoxygenase-derived products are acknowledged to be responsible for the hyper-reactivity of tracheal preparations treated with indomethacin or other cyclo-oxygenase inhibitors. Various lipoxygenase inhibitors have been shown to reverse the potentiating effects of indomethacin on contractile agonists (Mitchell 1982a, 1982b, 1982c, 1984). However leukotriene-induced contracture is believed to be of minor importance in comparison to inhibition of prostaglandin-induced relaxation (Jones et al., 1988).

The reduced relaxation of guinea-pig tracheal strips induced by NaNP following pre-incubation with indomethacin observed here contrasts with the inability of de-epithelialisation to reduce the potency of nitroglycerin reported by Goldie et al. (1986). Both relaxants exert their effects by stimulation of guanylate cyclase so similar results would have been expected. Of probable significance is the lack of effect of de-epithelialisation on ACh-induced contraction described by Goldie et al. (1986). The discrepancies observed with the nitrovasodilators are probably an extention of the differing results obtained with ACh and could demonstrate some fundamental experimental variance.

3.6.2a: EET-induced Relaxations in the Aorta

The discovery of a cytochrome P_{450} pathway for AA, producing a series of EETs, raises the question as to their role in the control of smooth muscle activity. Vasodilation has been demonstrated to 5(6)-, 8(9)- and 11(12)-EET in the rat intestinal microcirculation (Procter et al., 1987). In addition, during the course of this investigation, two further reports of EET-induced vasoactivity have been published (Carroll et al. 1987, 1988). Utilising the rat tail caudal artery, Carroll et al., demonstrated 5(6)-EET to be vasorelaxant, the observed activity being cyclo-oxygenase dependent. No vascular response was observed for the 8(9)-, 11(12)- and 14(15)-EETs.

In contrast to the findings of Carroll et al. (1987), all four EETs proved to be relaxant in the rat thoracic aorta. This discrepancy may be due to the use of different vascular preparations (the tail being a heat loss organ in the rat). Alternatively, the other EETs may have proved vasorelaxant in the caudal artery at concentrations greater than $2x10^{-6}M$. The 5(6)-isomer proved to be approximately 100 times more potent than the other isomers in the aorta. The greater potency of 5(6)-EET in the aorta is in accordance with the results observed in the intestinal microcirculation (Procter et al., 1987).

3.6.2b: EET-induced Relaxation in the Trachea

All four EETs were found to relax guinea-pig tracheal smooth muscle. Again the 5(6)-isomer was approximately 100 fold more potent than the other three epoxides, the potencies of which did not differ significantly in either tissue. However there were some important differences between EET-induced relaxations in tracheal and aortic preparations. The concentration of all four EETs required to induce relaxation in the trachea were 10 to 100 fold greater than in the aorta. Furthermore the maximal relaxation to 5(6)-EET in trachea was only 60% of maximal relaxation (NaNP-induced), whereas in the aorta 100% relaxation to 5(6)-EET was observed. 8(9)-, 11(12)- and 14(15)-EETs induced relaxations greater than 80% with respect to NaNP.

The greater potency of the 5(6)-EET in both aortic and tracheal preparations may be a result of its further conversion to a compound of greater potency. Indeed the 8,11,14 triene conformation preserved in 5(6)-EET following arachidonate epoxidation is known to be essential for cyclo-oxygenase substrates. This configuration is not preserved in the 8(9)-, 11(12)- and 14(15)-isomers. Indeed, Oliw (1984) has demonstrated the metabolism of 5(6)-EET by cyclo-oxygenase to prostaglandin epoxy-endoperoxides and to epoxy-PGE_1 and epoxy-$PGF_{1\alpha}$. The implications of further cyclo-oxygenase

and lipoxygenase conversions in EET-induced responses will be discussed later.

3.6.3: Endothelial Cell Involvement in EET-induced Relaxations of Rat Thoracic Aorta

Attenuation of the 5(6)-EET-induced response by the process of de-endothelialisation further supports the idea of conversion of 5(6)-EET to a compound with increased relaxant potency and would appear to locate this process to the endothelium. Preservation of the NaNP response militates against smooth muscle damage being the cause of the reduced relaxant effect. This was further supported by the absence of any alteration in 8(9)-, 11(12)- and 14(15)-EET-induced responses.

The fact that following de-endothelialisation the potency of 5(6)-EET did not revert back to the position of the other EETs may be explained by: 1) a small amount of endothelium, sufficient to maintain some conversion of 5(6)-EET, remained intact, 2) 5(6)-EET conversion may also take place in the smooth muscle.

In the rat caudal artery (Carroll et al., 1988), de-endothelialisation almost totally abolished 5(6)-EET-induced relaxation. This result differs from those reported here, the EC_{50} in denuded vessels was only twice that in intact preparations. This may demonstrate a greater role for the endothelial lining in 5(6)-EET-induced relaxation of the caudal artery than in thoracic

aorta.

3.6.4a: Effect of Indomethacin and NDGA on Aortic Relaxations Induced by the EETs

Exposure of aortic strips to $3x10^{-6}M$ indomethacin reduced the potency of 5(6)-EET to a similar degree as did the process of de-endothelialisation. Exposure to indomethacin had no inhibitory effect on 8(9)-, 11(12)- and 14(15)-EET induced responses. The reduced potency of 5(6)-EET in the presence of indomethacin suggests that a cyclo-oxygenase product of 5(6)-EET may contribute to the relaxant effects noted. Exposure to indomethacin was also reported to inhibit 5(6)-EET induced relaxation of the rat caudal artery (Carroll et al., 1988). The findings reported here in part agree with the proposal by Carroll et al., (1988) that the activity of 5(6)-EET requires an intact endothelium and further metabolism through cyclo-oxygenase. However, in the aorta 5(6)-EET also has a direct action of its own.

The inhibitory effect of indomethacin proved to be additive to that of de-endothelialisation. The concentration of 5(6)-EET required to induced relaxation in de-endothelialised aortic strips exposed to indomethacin was nearly the same as that of the other three EETs. The additive effects of both these treatments suggests that in isolation each treatment was not sufficient to completely block cyclo-oxygenase activity.

Further investigations utilising a higher concentration of indomethacin (say $1x10^{-5}M$) with or without de-endothelialisation may reduce the potency of 5(6)-EET to that of the other EETs. Alternatively 5(6)-EET be acting through a mechanism additional to cyclo-oxygenase which is located in the endothelium.

In accordance with the findings of Carroll et al. (1988), lipoxygenase inhibition by NDGA had no effect on 5(6)-EET induced relaxation. Furthermore, exposure to NDGA had no effect on the relaxations induced by the other EETs. These findings would indicate that the lipoxygenase pathway plays no role in EET-induced responses in vascular tissue.

3.6.4b: Effect of Indomethacin and NDGA on Tracheal Relaxations Induced by the EETs

Exposure of the trachea to indomethacin had a more pronounced inhibitory effect on the 5(6)-EET-induced relaxant response than observed in rat aorta. In contrast to the slight decrease in potency of 5(6)-EET in intact aorta, the potency of 5(6)-EET in trachea was reduced, becoming similar to that of the other EETs. The differing ability of indomethacin to attenuate 5(6)-EET-induced responses in aorta and trachea may be due to the different protocols used. Continual exposure to indomethacin in the tracheal rings could have reduced cyclo-oxygenase activity more effectively than the 5

minute pre-incubation allowed in the aortic strips. Continual exposure of the aortic preparations to indomethacin may attenuate 5(6)-EET responses to the same degree observed in tracheal rings.

The maximal response to 5(6)-EET in the trachea was increased in the presence of indomethacin. However in contrast, although the potencies of 8(9)-, 11(12)- and 14(15)-EET were not altered in the presence of indomethacin, the maximal responses observed were reduced by as much as 15%. This reduction in maximal relaxation to 8(9)-, 11(12)- and 14(15)-EET in the presence of indomethacin may be explained by the blockade of release of an inhibitory cyclo-oxygenase product by the epithelium (Butler et al., 1987). The increased response (but decreased potency) of 5(6)-EET in the presence of indomethacin supports the idea of two components to the 5(6)-EET response acting by different mechanisms. In the presence of indomethacin 5(6)-EET could act through the same mechanism as the other EETs, probably a direct action on the smooth muscle. De-epithelialised tracheal rings exposed to indomethacin would probably show similar responses to all four EETs.

From the work of Oliw (1984) the most likely product of 5(6)-EET metabolism by cyclo-oxygenase capable of inducing relaxation in the trachea would be epoxy-PGE_1. This correlates well with the findings of Coleman and Kennedy (1980) on the relaxant activity of PGE_1 in pre-contracted guinea-pig trachea. PGE_1 was found to be

relaxant over the same dose range as that identified for 5(6)-EET, the half maximal concentration for PGE_1 being $4.8x10^{-7}M$ compared to $1.3x10^{-6}M$ for 5(6)-EET. Furthermore the maximal relaxation observed with both PGE_1 and 5(6)-EET in ACh-precontracted guinea-pig trachea is 60% in both cases. However, there is no direct evidence regarding the potency of epoxy-PGE_1 in tracheal preparations.

As with aortic preparations, the involvement of the lipoxygenase pathway in EET-induced responses was militated against due to the absence of an effect with $1x10^{-6}M$ NDGA. The lack of any effect of NDGA on ACh-induced precontraction and NaNP-induced relaxation is of some interest. As previously discussed (section 3.6.1) in addition to an inhibitory cyclo-oxygenase product, the trachea has been shown to release a bronchoconstrictor lipoxygenase product (Mitchell, 1982a,b,c,d, 1984). However it should be noted that a reduction in ACh-induced response following exposure to NDGA was not observed, although exposure to indomethacin did increase the ACh-response. Furthermore in the presence of inhibitory cyclo-oxygenase products the leukotrienes play only a minor role (Jones et al., 1988). As a result any small change which may occur following lipoxygenase inhibition would be masked by the dominant cyclo-oxygenase effect. Exposure of indomethacin-treated tracheal rings to NDGA may indeed reverse the potentiaion of the ACh- and NaNP-induced responses. Alternatively the

concentration of NDGA utilised here may have been too low to produce any significant effects. However previous reports have shown $1x10^{-6}$M NDGA to effectively inhibit lipoxygenase activity in guinea-pig tracheal preparations (Yen, 1981; Burka, 1985; Chand et al., 1986).

3.6.5a: Effect of M&B 22,948 on Aortic Relaxations

The involvement of cGMP in aortic preparations was investigated utilising the cGMP-specific phosphodiesterase inhibitor M&B 22,948 (Weishaar et al., 1986). $3x10^{-6}$M of M&B 22,948 was observed to reduce the potency of PE in contracting aortic strips. Furthermore cGMP-phosphodiesterase inhibition was also found to augment the relaxant properties of ACh and NaNP. An understanding of the mechanism of action of EDRF readily explains these observations. As previously discussed, intact aortic strips release a basal level of EDRF (Griffith et al. 1984; Martin et al., 1985). Both EDRF- and nitrovasodilator-induced relaxations have been associated with increases in cGMP, and inhibition of cGMP formation and catabolism have also been shown to reduce and augment, respectively, such relaxant responses (Molina et al., 1987). Investigation of the role of cGMP in EET-induced relaxations would have been complicated by the basal release of EDRF. For this reason EET-induced relaxation was investigated using aortic strips denuded of their endothelial lining. Pre-incubation with M&B

22,948 potentiated the relaxation induced 8(9)-, 11(12)- and 14(15)-EET, but not to 5(6)-EET. This would suggest cGMP is not involved in the 5(6)-EET relaxatory mechanism but is important to the actions of the other EETs. However further investigation into the endothelial-dependent component of 5(6)-EET-induced relaxation may show a cAMP-dependence. The use of selective inhibitors of cAMP-phosphodiesterase such as Amipazone or MDL 17,043 (Weishaar et al., 1986) would be useful in evaluating such a role for cAMP.

3.6.5b: Effect of M&B 22,948 on Tracheal Relaxations

Exposure of tracheal preparations to M&B 22,948 prior to the addition of the EETs had an identical effect to that seen in aortic strips, i.e. augmentation of 8(9)-, 11(12)- and 14(15)-EET-induced relaxant responses, but not the 5(6)-EET-induced response. The absence of any effect on ACh-induced contraction indicates that cGMP is not of importance in the contractile response. As with the aorta, NaNP-induced relaxation was augmented in the presence of M&B 22,948. Investigations utilising cAMP-specific phosphodiesterase inhibitors would again verify any cAMP-dependent component in the 5(6)-EET relaxant response.

CHAPTER 4: ACTION OF THE EETs ON RAT BLOOD PLATELETS

4.1: INTRODUCTION

Platelets constitute an essential component of haemostasis which follows the disruption of the blood vessel wall. Aggregation of blood platelets forms a white thrombus which acts as the target for blood clot formation.

Stimulation of platelets by agonists, including thrombin, collagen, platelet aggregating factor (PAF), serotonin, vasopressin, arachidonic acid, dihomo-γ-linolenic acid, endoperoxide and thromboxane analogues, is associated with, and thought to proceed via PLC hydrolysis of inositol phospholipids (Siess, 1989). Receptor stimulation is believed to be linked to PLC-activation by guanine nucleotide-binding proteins (Haslam & Davidson, 1984; Brass et al., 1986). Conclusive information identifying the precise G-proteins responsible and the exact mechanisms involved has not been resolved. Activation of platelets by epinephrine and ADP appears to differ from other agonists, the mechanisms being uncertain.

Activation of PLC is known to be independent of intracellular Ca^{2+} (Billah & Lapetina, 1982b; Simon et al., 1984). Furthermore increasing intracellular Ca^{2+} levels by ionophores does not stimulate PLC (Imai & Nozawa, 1982; Rittenhouse-Simmons, 1981; Rittenhouse,

1984). PLC activity results in the formation of inositol 1,4,5-trisphophate (IP_3) and 1,2-diaylglycerol (DAG) (Rittenhouse-Simmons, 1979; Billah & Lapetina, 1982b).

Once liberated, DAG stimulates protein phosphorylation through the activation of protein kinase C (Nishizuka, 1984). Furthermore, increased levels of DAG are known to be necessary for granule secretion and exposure of fibrinogen receptors essential for aggregation (Shattil & Brass, 1987).

Increased IP_3 results in an increase in cytosolic calcium levels. Release from the dense tubular system is widely believed to account for the increase (Berridge & Irvine, 1984), however calcium release from PtdIns 4,5-P_2 upon hydrolysis would also be sufficient to account for the observed rise in cytosolic Ca^{2+} (Billah & Lapetina, 1982c). Increased levels of cytosolic Ca^{2+} are essential for platelet functioning; including shape change, secretion and fibrinogen receptor function (Siess, 1989). As a result, factors altering the level of cytosolic calcium would undoubtedly affect the aggregability of blood platelets.

To this end, cAMP inhibition of platelet activity is well documented (see Haslam et al., 1978). Inhibition of platelet function by cAMP is considered to be mediated by a reduction in cytosolic Ca^{2+} levels by an inhibition of PLC (Billah et al., 1979; Imai et al., 1983). Alternatively, increased levels of platelet cAMP, in the presence of a protein kinase, may reduce cytosolic Ca^{2+}

levels, and inhibiting platelet function, by stimulating calcium uptake into the dense tubular system (Kaser-Glanzmann et al., 1977).

Inhibition of PLC hydrolysis of PtdIns 4,5P_2 is proposed as the mechanism by which cGMP inhibits stimulus-induced increases in cytosolic Ca^{2+} levels (Brass & Laposata, 1987; Nakashima et al., 1986).

Metabolites of arachidonic acid can both stimulate or inhibit platelet activation. The prostaglandin endo-peroxides, G_2 and H_2, as well as thromboxane A_2 stimulate platelet aggregation. PGI_2, PGD_2, PGE_1, 12-HPETE and 12-HETE, on the other hand, increase levels of platelet cAMP and inhibit platelet aggregation.

Two reports describing cyclo-oxygenase inhibition by 8(9)-, 11(12)- and 14(15)-EET (Fitzpatrick et al. 1986, 1987) also demonstrated inhibition of AA-induced washed platelet aggregation by 8(9)-, 11(12)- and 14(15)-EET. The anti-aggregatory activity of these three epoxy-isomers as well as 5(6)-EET against ADP- and thrombin-induced aggregation are described here, together with results of cyclic nucleotide manipulation on EET-induced inhibition.

4.2: PREPARATION AND INCUBATION OF PLATELETS

Five 9ml aliquots of blood were taken from five separate Wistar rats using plastic syringes containing 1ml of trisodium citrate, to prevent clotting, giving a final citrate concentration of 0.38%. The whole blood was centrifuged in plastic tubes at 220g for twenty minutes. The supernatant was re-centrifuged at 220g for 10 minutes to remove any remaining red or white blood cells. The supernatant (platelet rich plasma, PRP) was removed without disturbing the buffy coat. If required, the PRP was then centrifuged at 1300g for 15 minutes to sediment the platelets. The platelet pellet was resuspended in a small volume (approximately 5ml) of 10mM HEPES-Tyrodes (pH 7.35) by gently aspirating the solution repeatedly with a plastic pasteur pipette. The resultant platelet suspension was finally diluted to 600,000 platelets/mm^3 $\pm$10%. A Coulter Thrombocounter was utilised to ascertain the platelet count.

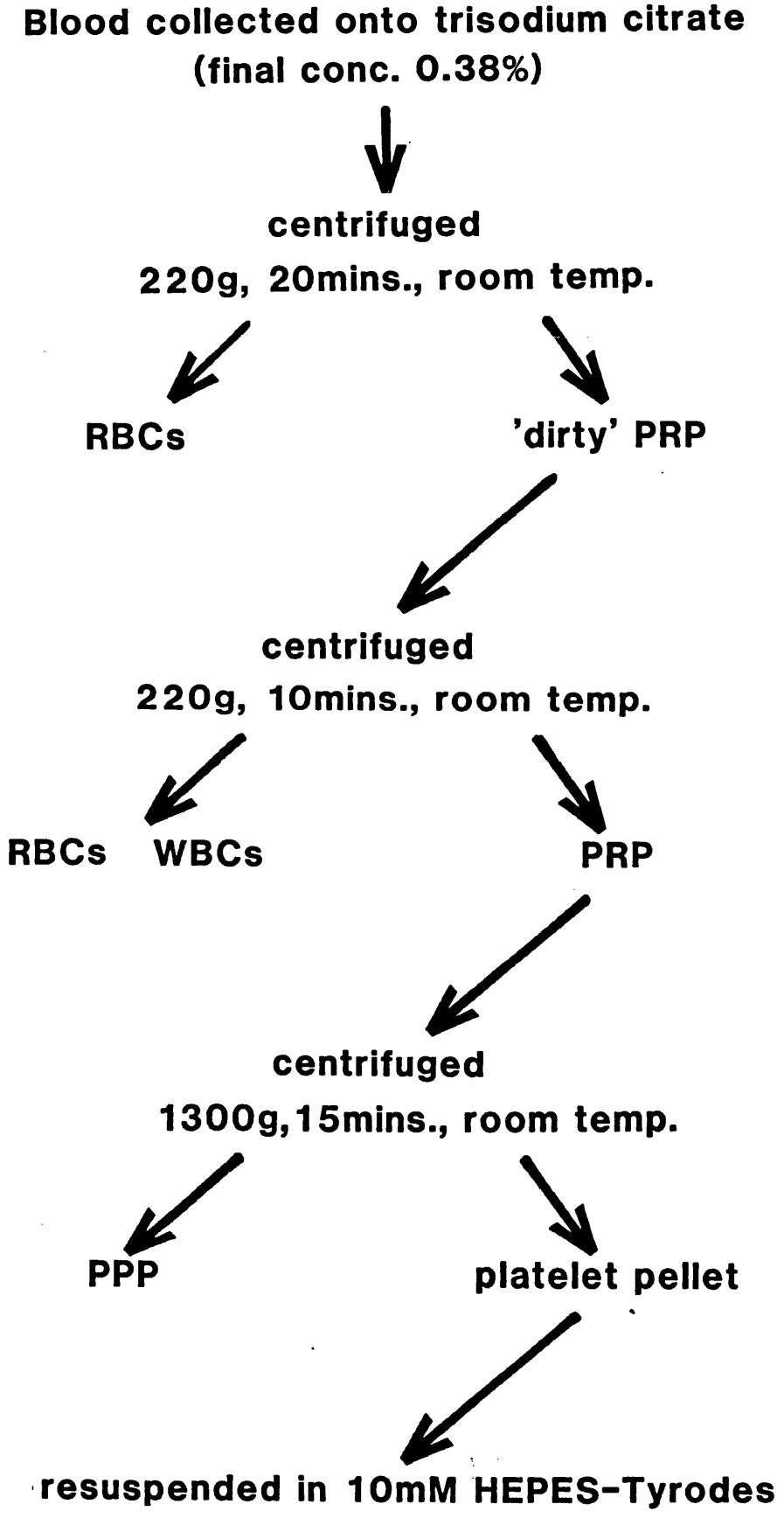
Platelet aggregation was investigated using 0.5ml aliquots of washed platelets or PRP in a Payton Aggregation Module (37°C, stirred at 500rpm). Changes in optical transmittance, indicating platelet aggregation, were recorded on a BBC SE120 chart recorder (chart speed 1cm/minute). The platelets were allowed to equilibrate in the aggregometer for two minutes both in the presence and absence of the epoxyeicosatrienoic acids. Volumes of added drugs were kept below 0.01ml to avoid possible

dilution effects. The effects of the EETs on both ADP- and thrombin-induced platelet aggregation in washed platelets, and on ADP-induced aggregation in PRP were investigated. Furthermore, anti-aggregatory effects of prostacyclin, PGE_1 and sodium nitroprusside (NaNP) on washed platelets were also examined as positive controls.

The possibility of *in situ* metabolism of the EETs was investigated by pre-incubation of the platelets for 30 seconds with either indomethacin (3×10^{-6}M) or NDGA (1×10^{-6}M) prior to the addition of EET.

Cyclic nucleotide involvement in platelet responses to the EETs, PGI_2 and NaNP were evaluated. 0.5ml aliquots of platelet suspension were pre-incubated for 30 seconds with either papaverine or M&B 22,948. A submaximal dose of EET, PGI_2 or NaNP was then introduced into the platelet suspension and the response compared to that in the absence of papaverine or M&B 22,948.

Unless otherwise stated, for statistical analysis Student's t-test for unpaired observations was used. When P was smaller than 0.05 values were considered to be significantly different.


Blood collected onto trisodium citrate
(final conc. 0.38%)
centrifuged
220g, 20mins., room temp.
RBCs
'dirty' PRP
centrifuged
220g, 10mins., room temp.
RBCs
WBCs
PRP
centrifuged
1300g,15mins., room temp.
PPP
platelet pellet
resuspended in 10mM HEPES-Tyrodes

4.3: DETERMINATION OF EET AND PGI_2 HALF-RESPONSE TIMES

Blood was taken from Wistar rats as above and the platelets separated by the same procedure. For the purpose of determination of half-response time, the platelets were diluted to 750,000 platelets/mm^3 $\pm$10%. The greater concentration of platelets in this instance allowed 0.1ml of Hepes-Tyrodes to be added to the aggregometer cuvette prior to the platelet suspension, as incubation medium for the test compounds. On addition of 0.4ml of the platelet suspension, the final platelet count would be 600,000 platelets/mm^3 $\pm$10% as used above.

Doses of the test compounds (EETs and PGI_2) giving 100% inhibition of platelet aggregation were added to the Hepes-Tyrodes at 37°C. After a predetermined time had elapsed (0-180 seconds for PGI_2 and 0-25 minutes for EET determinations), 400ul of platelet suspension was introduced to the cuvette followed by the dose of ADP known to give 100% aggregation.

By observing the time-dependent decrease in anti-aggregatory activity of the test compounds a biological half-response time could be calculated.

In addition an approximate chemical half-life was also determined from the half-response time results. This was achieved utilising the linear portion of a dose-response curve (between 30% and 70% of observed maximal responses). The concentration giving 70% inhibition of platelet aggregation was determined from the individual

inhibitory dose/response curves, and the respective time for this response from the half-response/time plots noted (time 1). The expected inhibitory response that would be observed for half this concentration of drug was also determined from the individual inhibitory dose/response curves. The time after which this response would be observed was extrapolated from the half-response/time plot (time 2). By subtracting time 1 from time 2 an approximation of the time taken for 50% chemical decomposition could be determined.

4.4: RESULTS

4.4.1: Effect of ADP and EETs on Platelet Rich Plasma

Aggregation of platelet rich plasma (PRP) was induced by ADP in the range 4×10^{-6}M to 3×10^{-5}M (EC_{50} 1.3 $\pm$ 0.1 x 10^{-5}M (n=7)) (Fig. 4.1). The time taken for complete aggregation was less than two minutes. A concentration of 3×10^{-5}M ADP was sufficient to maintain peak aggregation for longer than 1 minute.

All four epoxyeicosatrienoic acids were found to inhibit platelet aggregation in PRP (Fig. 4.2). The 5(6)-isomer was significantly ($P<0.001$) more potent an inhibitor of aggregation than the remaining three isomers. Inhibition of PRP aggregation by 5(6)-EET was observed in the range 1×10^{-6}M to 3×10^{-5}M (IC_{50} 1.4 $\pm$ 0.1 x 10^{-5}M (n=4)). The 8(9)-, 11(12)- and 14(15)- isomers inhibited ADP-induced aggregation of PRP in the range 1×10^{-5}M to 2×10^{-4}M (IC_{50} values of 8.2 $\pm$ 0.6 x 10^{-5}M (n=4), 5.7 $\pm$ 0.3 x 10^{-5}M (n=4) and 5.7 $\pm$ 0.5 x 10^{-5}M (n=4) respectively). Inhibition of ADP-induced aggregation by 8(9)-, 11(12)- and 14(15)-EET showed no significant variation between the isomers ($P>0.05$).

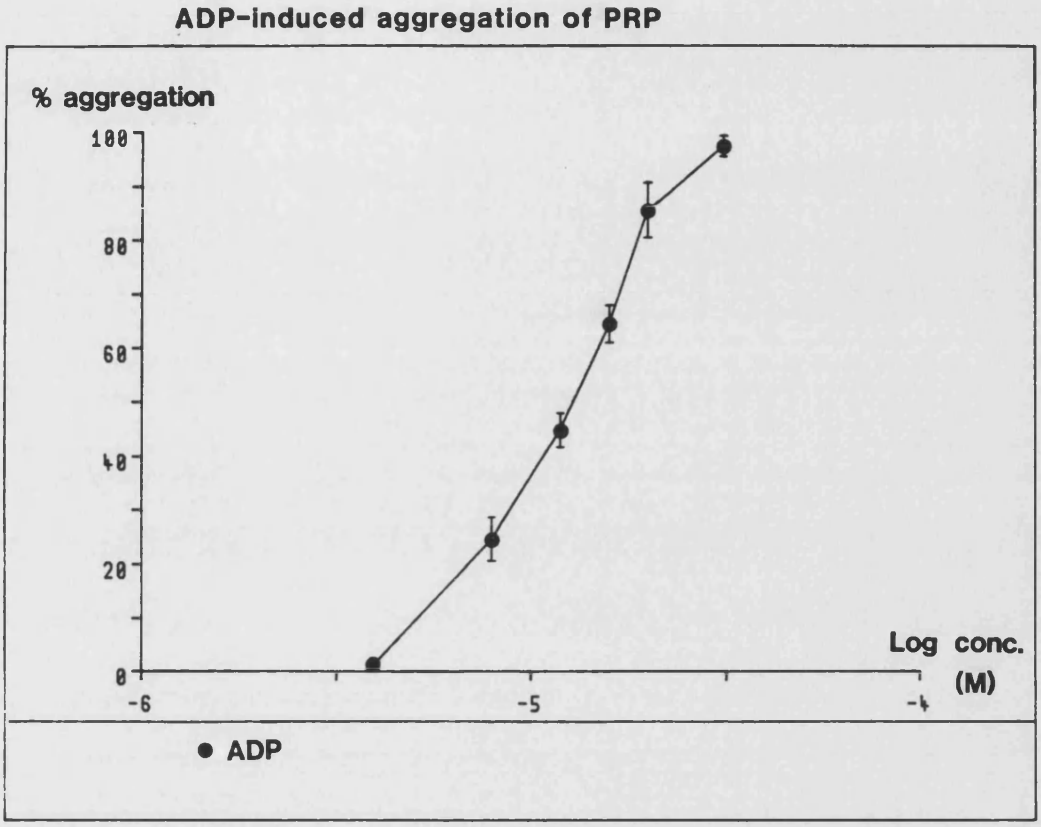


FIGURE 4.1
ADP-induced aggregation of platelet rich plasma. 0.5ml aliquots of PRP at 37°C were aggregated by ADP in the range $4x10^{-6}$ to $3x10^{-5}$M (EC_{50} of 1.3 ± 0.1 x 10^{-5}M). Values are expressed as a mean ± SEM (n=7).

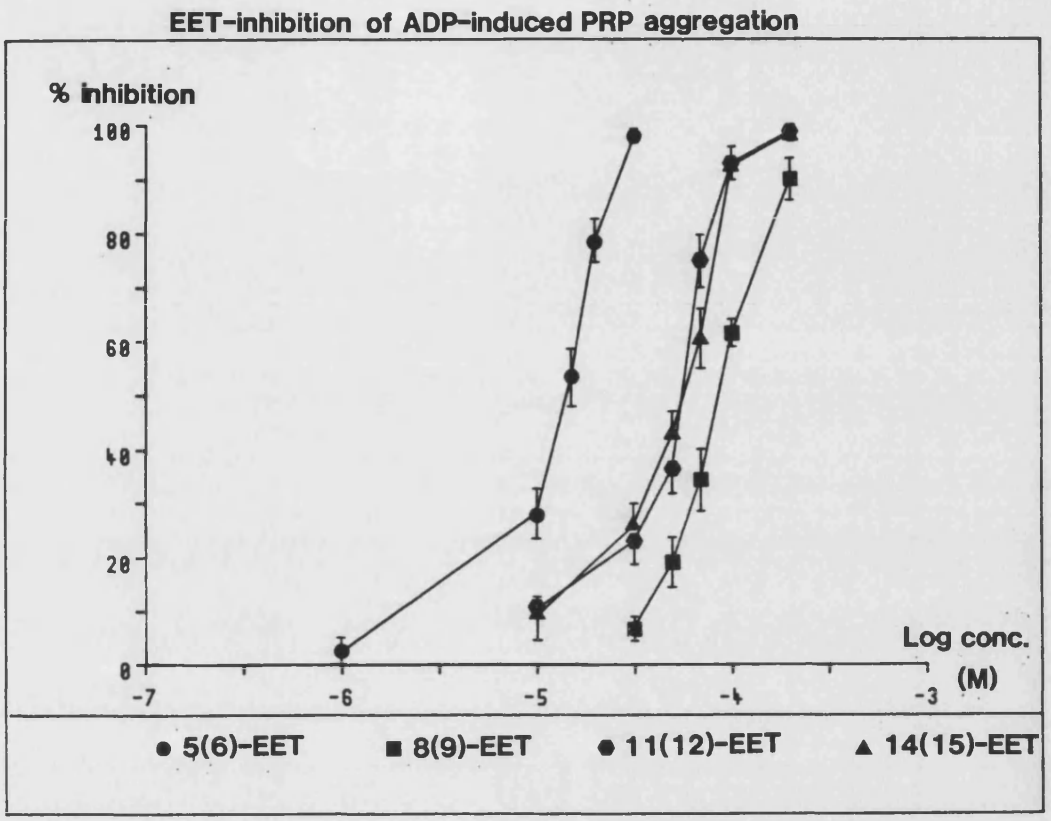


FIGURE 4.2

EET-inhibition of ADP-induced aggregation of platelet rich plasma. 0.5ml aliquots of PRP were pre-incubated with EET for 2 minutes at 37°C prior to the addition of ADP. All four EETs inhibited aggregation at micromolar concentrations. IC_{50} values were 1.4 $\pm$ 0.1 x 10^{-5}M for 5(6)-EET, 8.2 $\pm$ 0.6 x 10^{-5}M for 8(9)-EET, 5.7 $\pm$ 0.3 x 10^{-5}M for 11(12)-EET and 5.7 $\pm$ 0.5 x 10^{-5}M for 14(15)-EET. Values are expressed as a mean $\pm$ SEM (n=4).

4.4.2: Effect of ADP, Thrombin and EETs on Washed Platelets

ADP was found to be nearly ten fold more potent in inducing aggregation of washed platelets than PRP (Fig. 4.3). Aggregation was observed between $3x10^{-7}M$ and $3x10^{-6}M$ ADP (EC_{50} 4.8 $\pm$ 0.1 x $10^{-7}M$ (n=10)). Thrombin-induced aggregation of washed platelets was observed between 0.05 and 0.85 NIHunits/ml (EC_{50} 0.349 $\pm$ 0.019 NIHunits/ml (n=8)) (Fig. 4.4).

EET inhibition of ADP-induced aggregation in washed platelets was 10-fold greater than in PRP. Inhibition of ADP-induced platelet aggregation by 5(6)-EET was observed between $1x10^{-7}M$ and $2x10^{-6}M$ (IC_{50} value 4.2 $\pm$ 1.0 x 10^{-7} (n=4)) (Figure 4.6). 5(6)-EET was significantly more active ($P<0.005$) than 8(9)-, 11(12)- and 14(15)-EET which were roughly equipotent ($P>0.60$), inhibiting aggregation in the range $1x10^{-6}M$ to $2x10^{-5}M$. IC_{50} values for 8(9)-, 11(12)- and 14(15)-EETs were 6.6 $\pm$ 1.9 x $10^{-6}M$ (n=3), 6.7 $\pm$ 2.0 x 10^{-6} (n=3) and 5.7 $\pm$ 1.1 x $10^{-6}M$ (n=3) respectively.

With thrombin as the aggregatory agent (Fig. 4.7), 5(6)-EET was the most potent anti-aggregatory of the four isomers ($P<0.001$), with an effective dose range of $1x10^{-7}M$ to $2x10^{-6}M$ (IC_{50} value: 6.7 $\pm$ 1.9 x $10^{-7}M$ (n=4)). The other EET-isomers were approximately 30-100 fold less potent than 5(6)-EET, inhibiting thrombin-induced aggregation in the range $5x10^{-6}M$ to

$5x10^{-5}M$. IC_{50} values for the 8(9)-, 11(12)- and 14(15)-EETs were 4.0 $\pm$ 0.8 x $10^{-5}M$ (n=3), 2.2 $\pm$ 0.3 x $10^{-5}M$ (n=3) and 1.6 $\pm$ 0.5 x $10^{-5}M$ (n=3) respectively.

Whilst the 5(6)-EET was equipotent at inhibiting both thrombin-induced and ADP-induced aggregation (Fig. 4.5), 8(9)-, 11(12)- and 14(15)-EET were about ten fold less inhibitory against thrombin-induced platelet aggregation than against ADP.

Examination of the platelet pre-incubation period with the EETs revealed the inhibitory effect of the EETs to be immediate. Reduction of the pre-incubation period had no effect on the degree of inhibition obtained for any particular EET- concentration. It was therefore concluded that inhibitory doses of EET could be introduced to the platelet suspension just prior to the agonist.

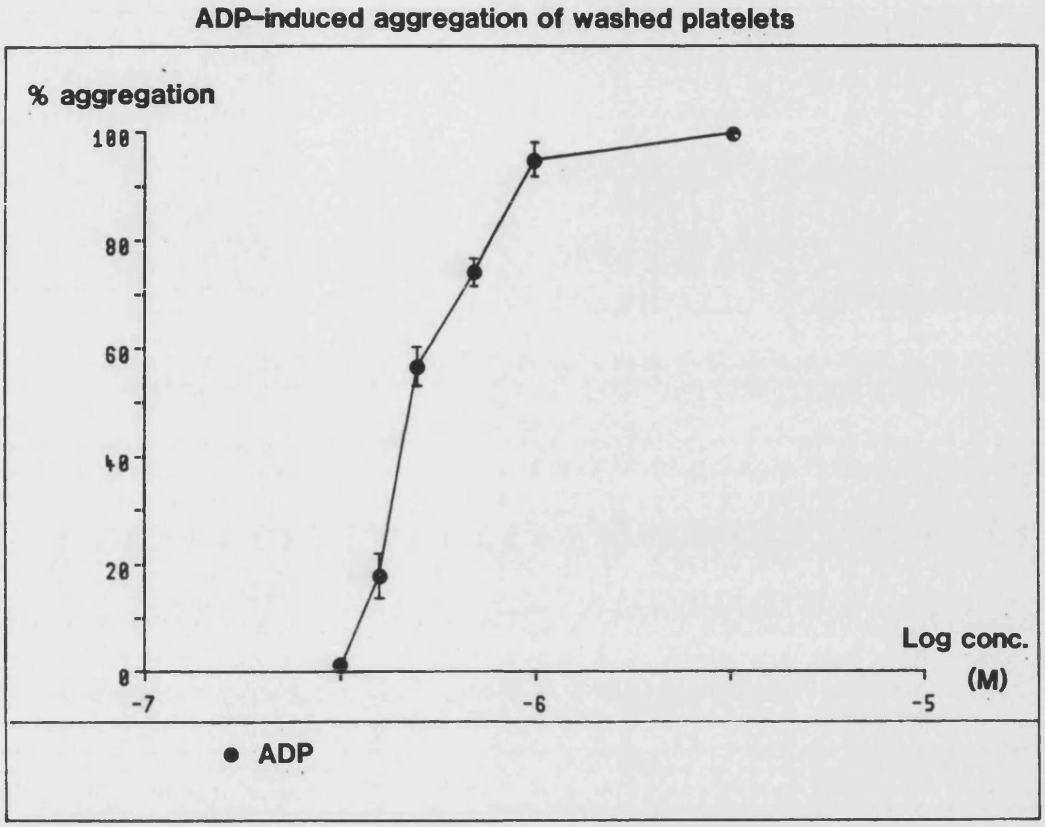


FIGURE 4.3

ADP-induced aggregation of washed platelets. 0.5ml aliquots of washed platelets were aggregated by ADP at 37°C. ADP induced aggregation in the range $3x10^{-7}M$ to $3x10^{-6}M$ (EC_{50} of 4.8 $\pm$ 0.1 x $10^{-7}M$). Values are expressed as a mean $\pm$ SEM (n=10).

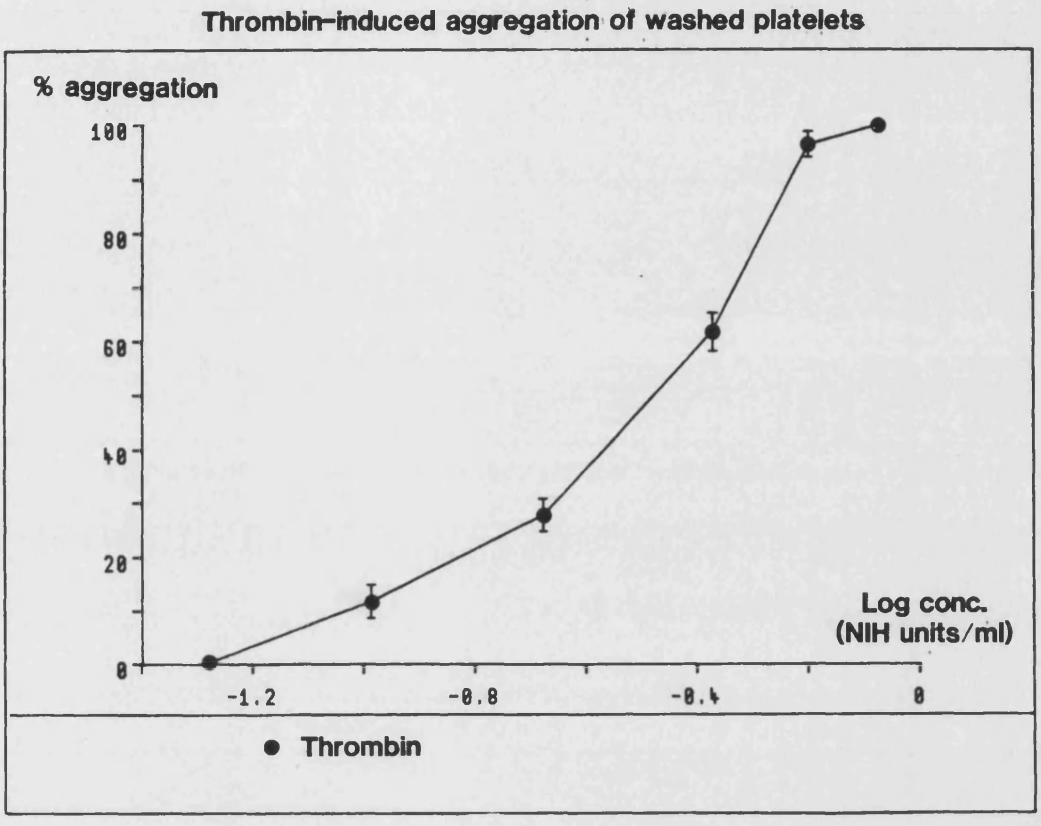
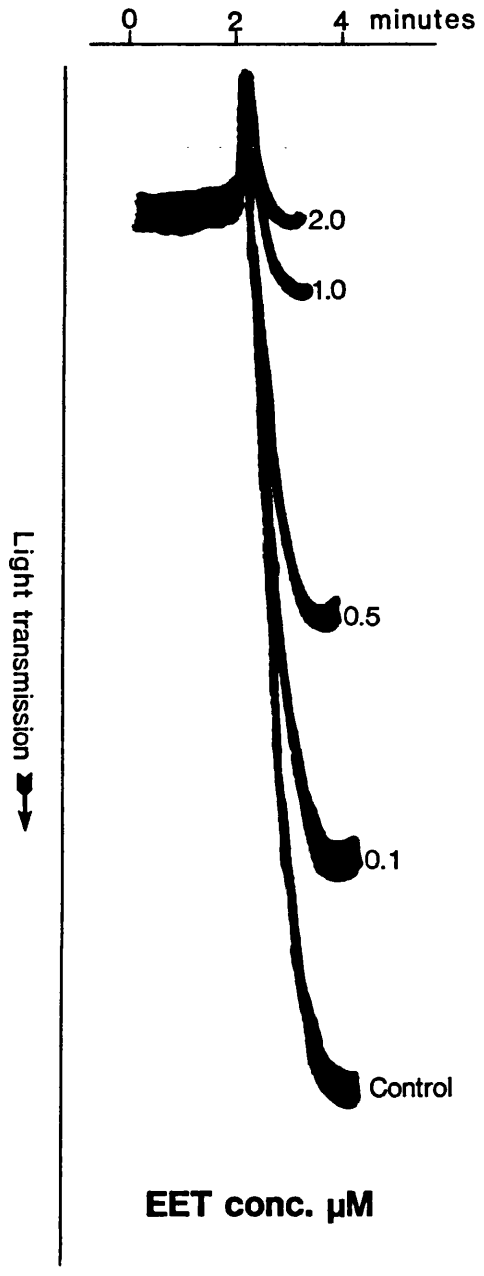


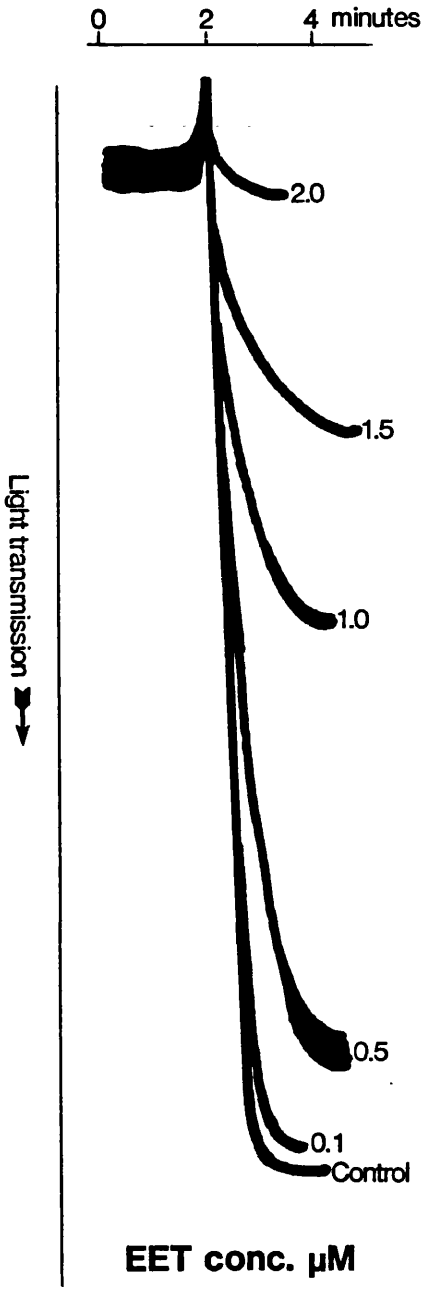
FIGURE 4.4

Thrombin-induced aggregation of washed platelets. 0.5ml aliquots of washed platelets were aggregated by thrombin at 37°C. Thrombin induced aggregation in the range 0.05 to 0.85 NIHunits/ml (EC_{50} of 0.35 $\pm$ 0.02 NIHunits/ml). Values are expressed as a mean $\pm$ SEM (n=8).

FIGURE 4.5

Representative traces of (a) 5(6)-EET inhibition of ADP-induced washed platelet aggregation and (b) 5(6)-EET inhibition of thrombin-induced washed platelet aggregation. Readings have been superimposed.


0
2
4 minutes
2.0
1.0
0.5
0.1
Control
Light transmission
EET conc. µM


0
2
4 minutes
2.0
1.5
1.0
0.5
0.1
Control
Light transmission
EET conc. µM

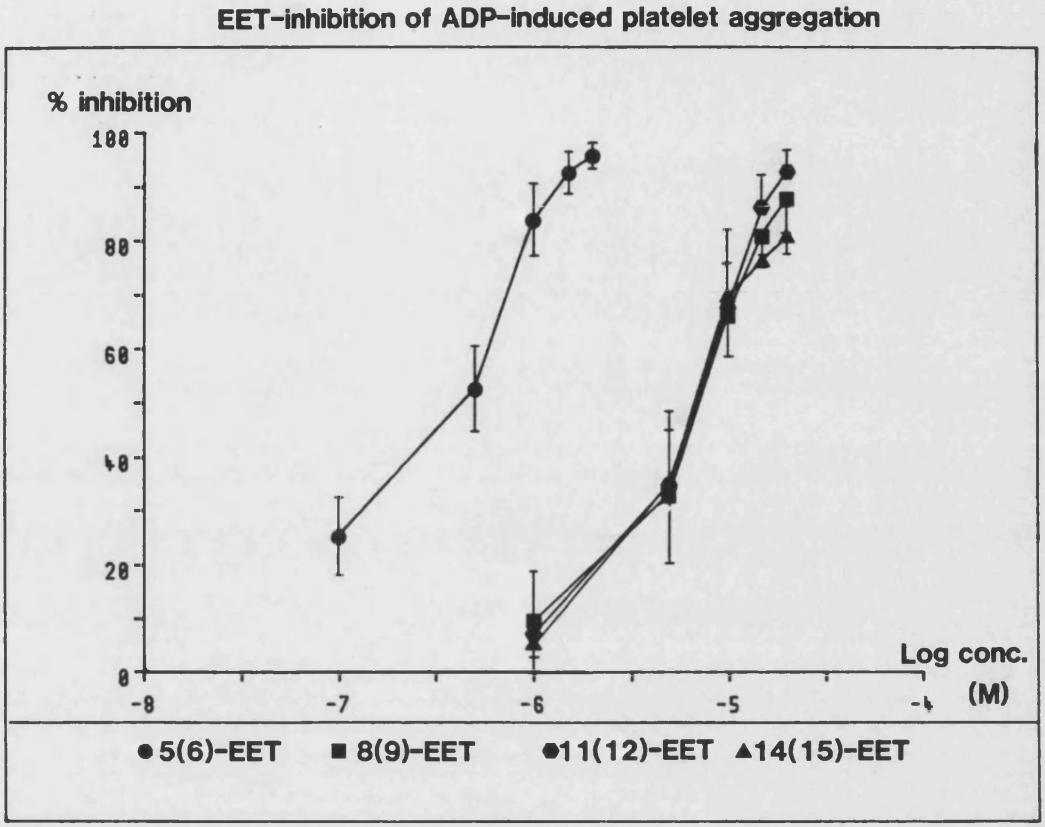


FIGURE 4.6

EET-inhibition of ADP-induced aggregation of washed platelets. 0.5ml aliquots of washed platelets were pre-incubated with EET for 2 minutes at 37°C prior to the addition of ADP. All four EETs inhibited aggregation at approximately ten fold lower concentrations than observed in PRP. IC_{50} values were 4.2 $\pm$ 0.1 x 10^{-7}M for 5(6)-EET, 6.6 $\pm$ 1.9 x 10^{-6}M for 8(9)-EET, 6.7 $\pm$ 2.0 x 10^{-6}M for 11(12)-EET and 5.7 $\pm$ 1.1 x 10^{-6}M for 14(15)-EET. 5(6)-EET was significantly more potent than the other EETs ($P<0.01$). Values are expressed as a mean $\pm$ SEM (n=4 for 5(6)-EET and n=3 for 8(9)-, 11(12)- and 14(15)-EETs).

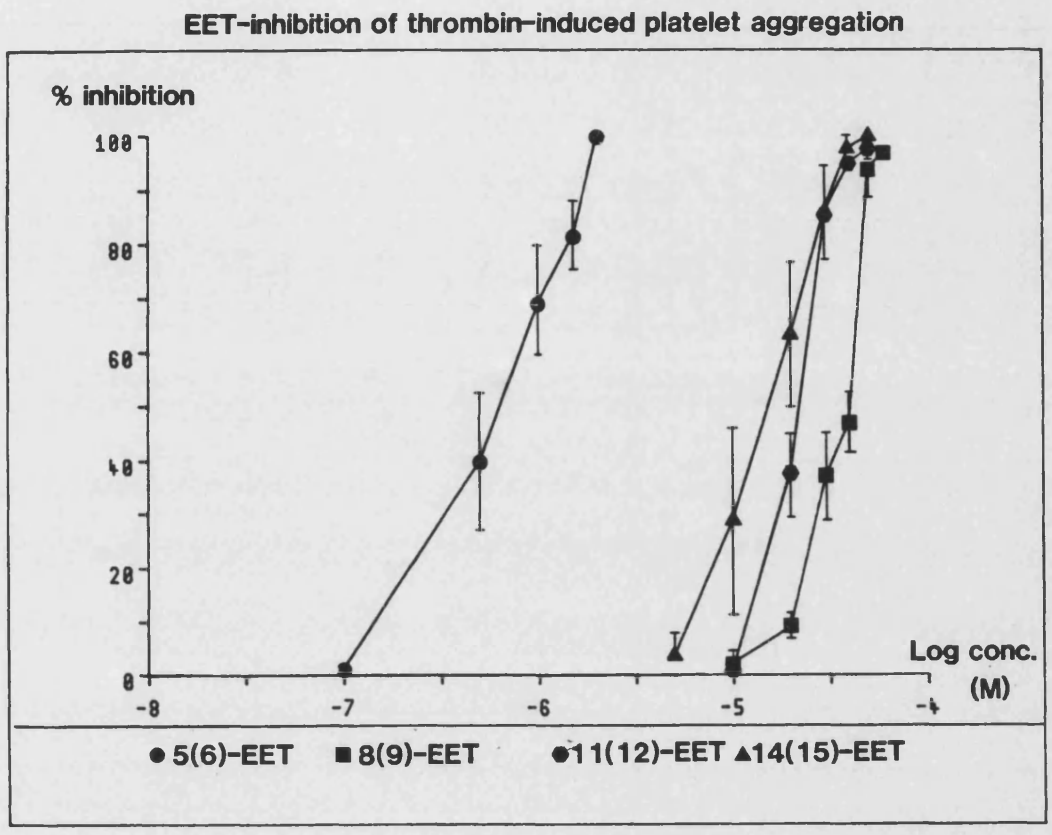


FIGURE 4.7

EET-inhibition of thrombin-induced aggregation of washed platelets. 0.5ml aliquots of washed platelets were pre-incubated with EET for 2 minutes at 37°C prior to the addition of thrombin. All four EETs inhibited aggregation. IC_{50} values were 6.7 $\pm$ 1.9 x 10^{-7}M for 5(6)-EET, 4.0 $\pm$ 0.8 x 10^{-5}M for 8(9)-EET, 2.2 $\pm$ 0.3 x 10^{-5}M for 11(12)-EET and 1.6 $\pm$ 0.5 x 10^{-5}M for 14(15)-EET. 5(6)-EET was again the most potent of the EETs. Values are expressed as a mean $\pm$ SEM (n=4 for 5(6)-EET and n=3 for 8(9)-, 11(12)- and 14(15)-EETs).

4.4.3: Effect of Indomethacin and NDGA on EET-inhibition of Washed Platelet Aggregation

To investigate whether platelet cyclo-oxygenase or lipoxygenase enzymes contributed to the EET-induced inhibition of aggregation, the platelet suspensions were pre-incubated with either indomethacin or NDGA for 30 seconds prior to EET addition.

The maximal dose of indomethacin or NDGA which could be pre-incubated with washed platelets without altering the observed ADP-induced aggregation was ascertained. Concentrations of $3x10^{-6}M$ indomethacin and $1x10^{-6}M$ NDGA could be incorporated into washed platelet suspensions without attenuating the aggregation induced by ADP. Higher concentrations of both compounds were found to inhibit ADP-induced platelet aggregation.

Figure 4.8 shows representative platelet aggregation traces in which 5(6)-EET inhibitory effects are augmented by exposure of the platelets to either indomethacin or NDGA.

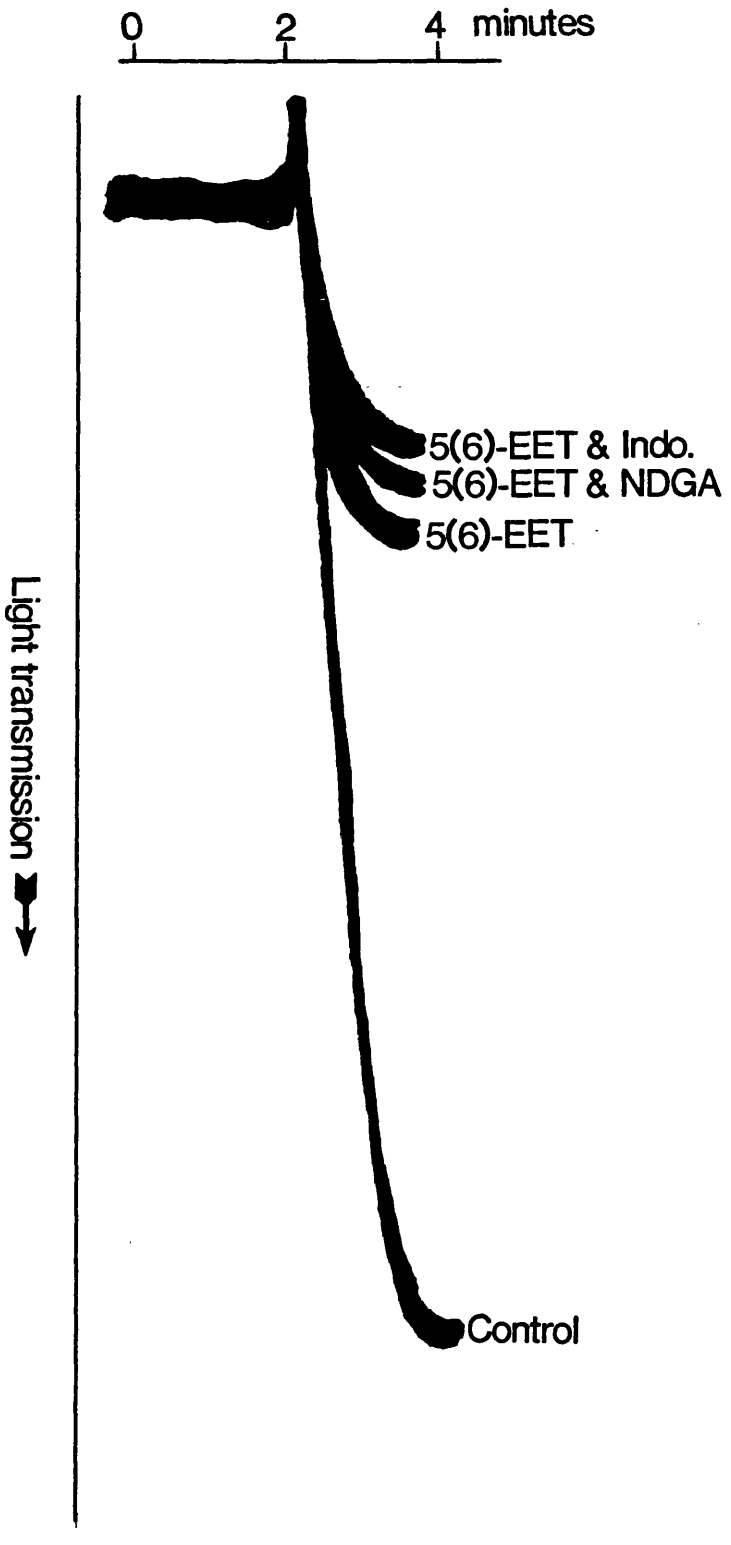
Pre-incubation of the washed platelets with $3x10^{-6}M$ indomethacin for 30 seconds at 37°C prior to the addition of the EETs potentiated the anti-aggregatory properties of the EETs (Fig. 4.9). Mean changes in percentage inhibitory activity of the 5(6)-, 8(9)-, 11(12)- and 14(15)-EETs were 15.8 $\pm$ 11.0 (n=3), 11.3 $\pm$ 4.2 (n=3), 7.1 $\pm$ 3.5 (n=3) and 6.3 $\pm$ 2.4 (n=3) respectively. Although the potentiations were found to be consistent they were

not statistically significant as determined by the students t-test of paired data ($P>0.10$). The maximal dose of indomethacin which could be utilised without affecting the ADP-induced response was not sufficient to completely block AA-induced platelet aggregation.

Pre-incubation of the washed platelets for 30 seconds at 37°C with the lipoxygenase inhibitor NDGA ($1x10^{-4}M$) also potentiated the anti-aggregatory effects of the EETs (Fig. 4.10). Mean percentage changes were 14.7 ± 6.2 (n=3), 5.7 ± 1.1 (n=3), 19.9 ± 2.9 (n=3) and 9.2 ± 1.8 (n=3) respectively. The potentiations were not statistically significant ($P>0.05$), as determined by Student's t-test of paired data.

Neither indomethacin or NDGA, at the concentrations used, had any effect on ADP-induced platelet aggregation.

FIGURE 4.8
Representative traces of indomethacin and NDGA augmentation of 5(6)-EET inhibition of ADP-induced washed platelet aggregation.


0
2
4 minutes
5(6)-EET & Indo.
5(6)-EET & NDGA
5(6)-EET
Control
Light transmission

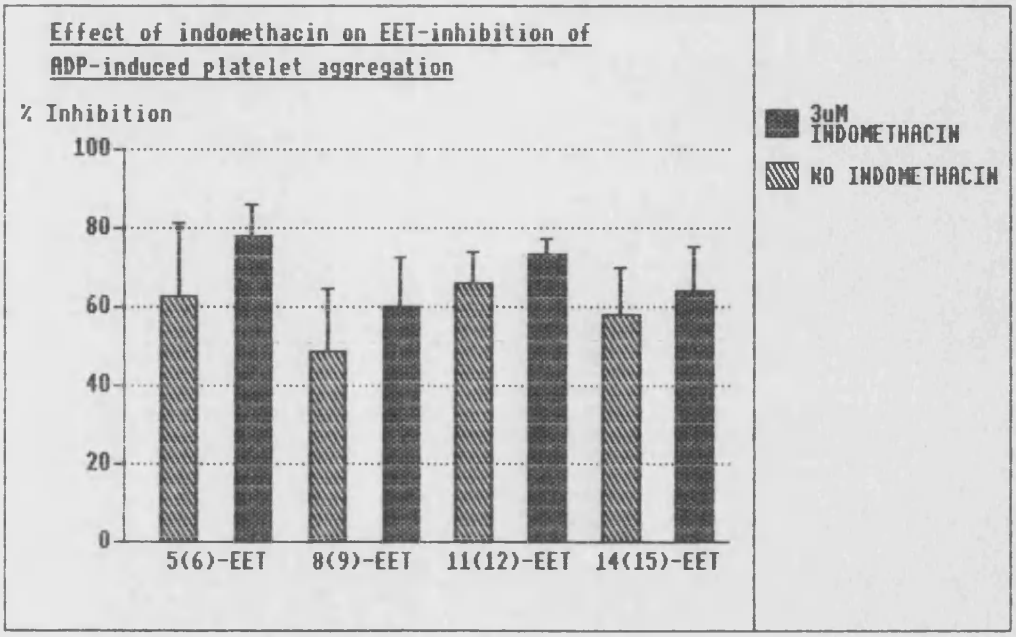


FIGURE 4.9

Effect of $3x10^{-6}$M indomethacin on submaximal EET inhibition of ADP-induced washed platelet aggregation. 0.5ml aliquots of washed platelets were pre-incubated with $3x10^{-6}$M indomethacin at 37°C for 30 seconds prior to the addition of EET. Following a 30 second pre-incubation with a sub-maximal dose of EET the platelets were aggregated with ADP. Inhibitory effects of all four EETs were augmented in the presence of indomethacin. Mean percentage increases in inhibition were 15.8 $\pm$ 11.0 for 5(6)-EET, 11.3 $\pm$ 4.2 for 8(9)-EET, 7.1 $\pm$ 3.5 for 11(12)-EET and 6.3 $\pm$ 2.4 for 14(15)-EET. Values are expressed as a mean $\pm$ SEM (n=3).

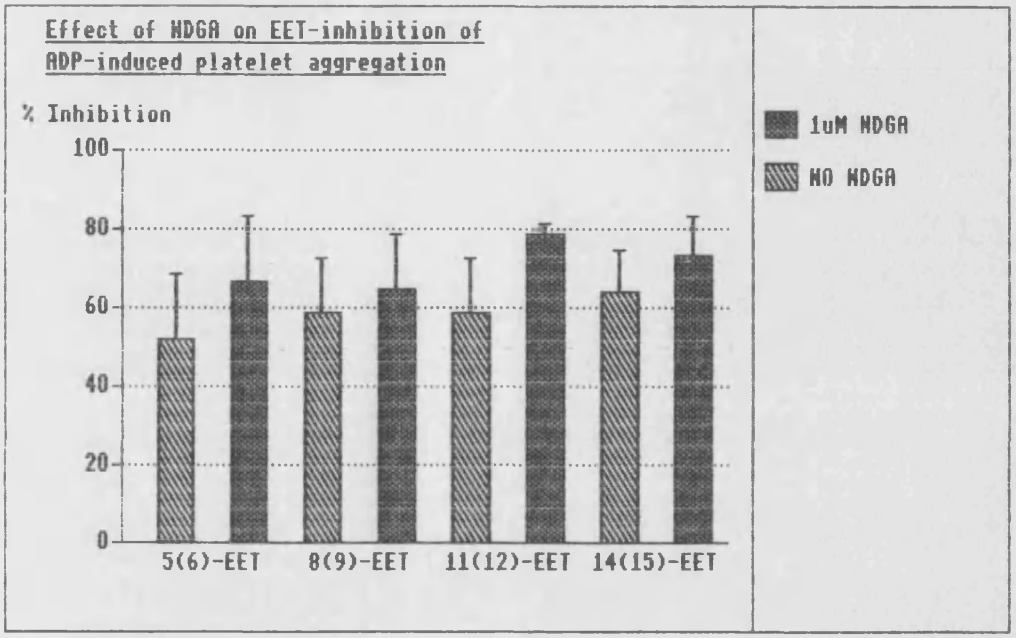


FIGURE 4.10

Effect of $1x10^{-6}$M NDGA on submaximal EET inhibition of ADP-induced washed platelet aggregation. 0.5ml aliquots of washed platelets were pre-incubated with $1x10^{-6}$M NDGA at 37°C for 30 seconds prior to the addition of EET. Following a 30 second pre-incubation with a sub-maximal dose of EET the platelets were aggregated with ADP. Inhibitory effects of all four EETs were augmented in the presence of NDGA. Mean percentage increases in inhibition were 14.7 $\pm$ 6.2 for 5(6)-EET, 5.7 $\pm$ 1.1 for 8(9)-EET, 19.9 $\pm$ 2.9 for 11(12)-EET and 9.2 $\pm$ 1.8 for 14(15)-EET. Values are expressed as a mean $\pm$ SEM (n=3).

4.4.4: Effect of M&B 22,948 on EET-inhibition of Washed Platelet Aggregation

To obtain some insight into the involvement of cyclic nucleotides in the inhibition of platelet aggregation by epoxyeicosatrienoic acids, papaverine and M&B22,948 were pre-incubated with the platelets prior to the introduction of the EETs. PGI_2 and NaNP were used as positive controls to verify that an adequate concentration of phosphodiesterase inhibitor had been used to enhance cAMP or cGMP mediated responses.

PGI_2 was found to be potently anti-aggregatory, exceeding 5(6)-EET-induced inhibition 200 fold (Fig. 4.12). PGI_2 inhibition of ADP-induced washed platelet aggregation was observed between $3x10^{-10}$ to $3x10^{-8}$M with an IC_{50} value of $3.5 \pm 0.4 \times 10^{-9}$M (n=6). NaNP inhibited washed platelet aggregation in the range $3x10^{-6}$M to $1x10^{-3}$M (IC_{50} $3.9 \pm 0.8 \times 10^{-5}$M (n=8) (Fig. 4.13).

PGE_1 was also found to be more potent than 5(6)-EET (Fig. 4.11). PGE_1 inhibited ADP-induced aggregation in the range $1x10^{-9}$ to $3x10^{-7}$M and had an IC_{50} value of $1.0 \pm 0.5 \times 10^{-8}$M (n=4).

Pre-incubation of washed platelets with $3x10^{-6}$M M&B 22,948 had no significant effect on EET-inhibition of ADP-induced platelet aggregation ($P>0.10$) (Fig. 4.15). NaNP-induced inhibition was significantly ($P<0.001$) augmented by the same concentration of M&B 22,948. The mean change in NaNP-induced inhibition was $43.0 \pm 2.1\%$

(n=4) compared with the mean changes for 5(6)-, 8(9)-, 11(12)- and 14(15)-EET-induced inhibition of 1.3 ± 1.0%, 0.5 ± 1.6%, 1.3 ± 1.2% and 2.0 ± 1.0% respectively (all n=4). ADP-induced aggregations were not effected by $3x10^{-6}$M M&B 22,948.

Papaverine ($3x10^{-6}$M), a non-selective phosphodiesterase inhibitor, augmented PGI_2-inhibition of ADP-induced aggregation (Fig. 4.14). The mean change in PGI_2-induced inhibition was 32.8 ± 0.9% (n=4). EET-induced inhibitions were also augmented by papaverine, mean changes for the 5(6)-, 8(9)-, 11(12)- and 14(15)-EETs were 31.5 ± 1.3%, 17.0 ± 1.1%, 43.3 ± 1.7% and 24.3 ± 0.6% respectively (all n=4). The increase in inhibitory activity of the EETs and PGI_2 in the presence of $3x10^{-6}$M papaverine was statistically significant $P<0.001$, (Student's t-test on paired data).

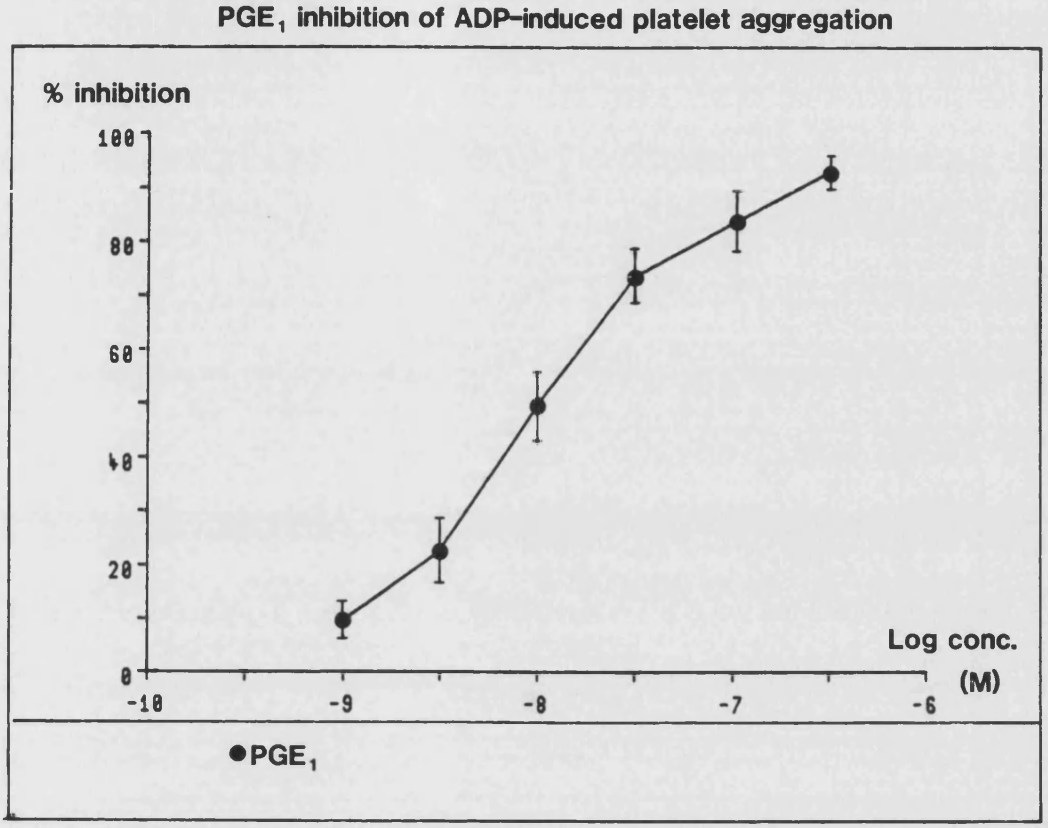


FIGURE 4.11

PGE_1-inhibition of ADP-induced aggregation of washed platelets. 0.5ml aliquots of washed platelets were pre-incubated with PGE_1 for 30 seconds at 37°C prior to the addition of ADP. PGE_1 inhibited aggregation in the range $1x10^{-9}M$ to $3x10^{-7}M$ (IC_{50} of 1.0 $\pm$ 0.5 x $10^{-8}M$). Values are expressed as a mean $\pm$ SEM (n=4).

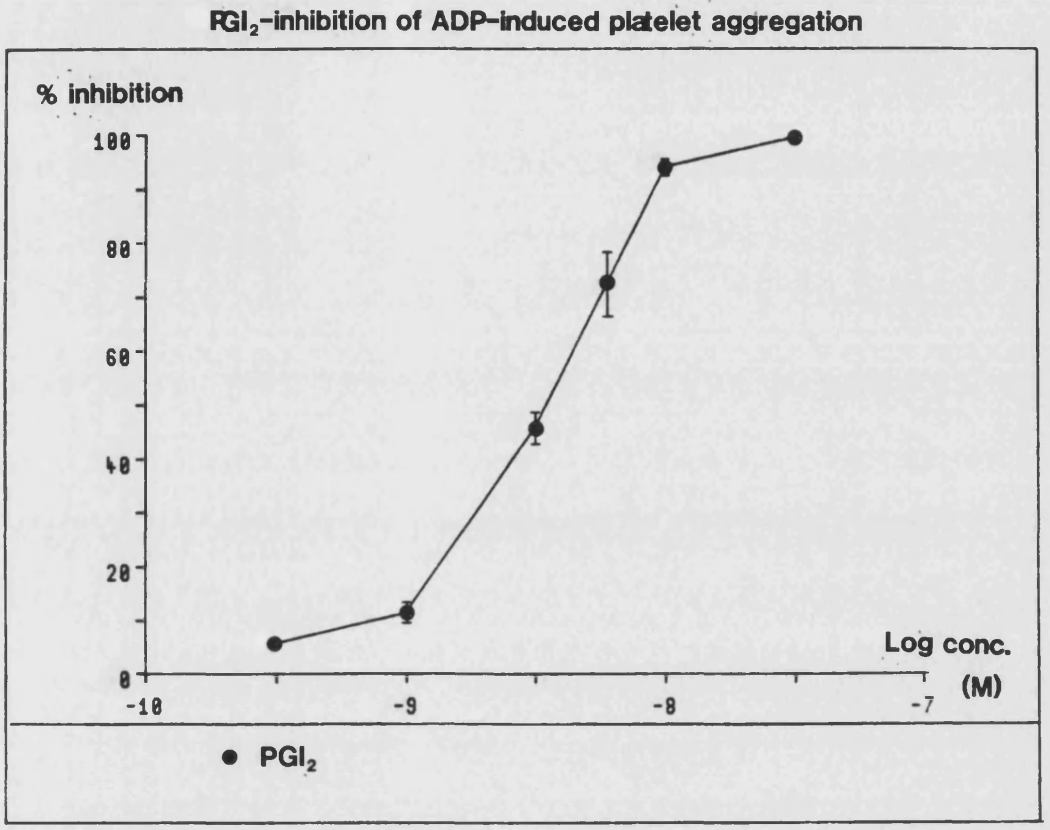


FIGURE 4.12

PGI_2-inhibition of ADP-induced aggregation of washed platelets. 0.5ml aliquots of washed platelets were pre-incubated with PGI_2 for 30 seconds at 37°C prior to the addition of ADP. PGI_2 inhibited aggregation in the range $3x10^{-10}M$ to $3x10^{-8}M$ (IC_{50} of 3.5 $\pm$ 0.4 x $10^{-9}M$). Values are expressed as a mean $\pm$ SEM (n=6).

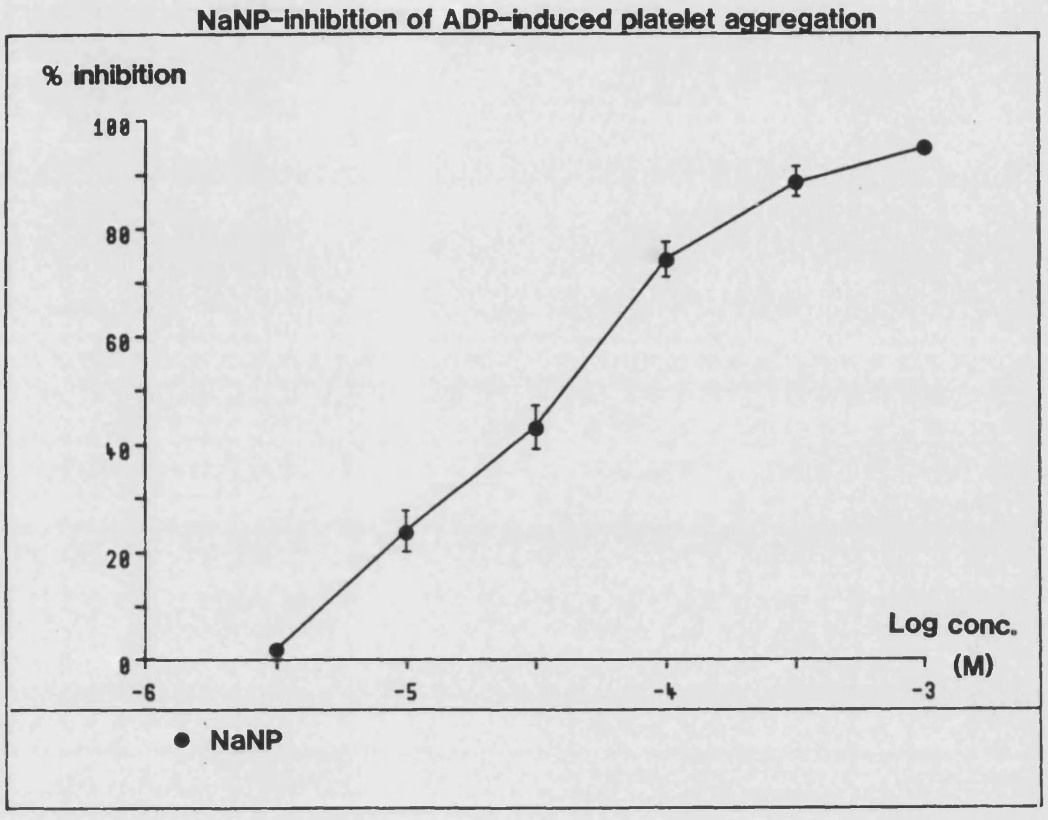


FIGURE 4.13

NaNP-inhibition of ADP-induced aggregation of washed platelets. 0.5ml aliquots of washed platelets were pre-incubated with NaNP for 30 seconds at 37°C prior to the addition of ADP. NaNP inhibited aggregation in the range 3×10^{-6}M to 1×10^{-3}M (IC_{50} of 3.9 $\pm$ 0.8 $\times 10^{-5}$M). Values are expressed as a mean $\pm$ SEM (n=8).

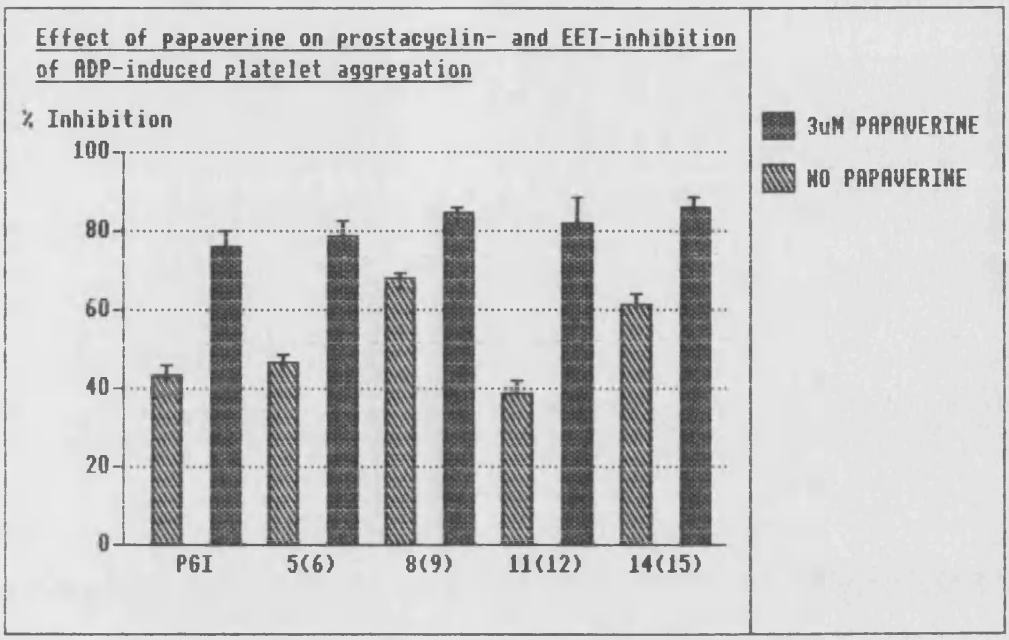


FIGURE 4.14

Effect of $3x10^{-6}$M papaverine on submaximal EET and PGI_2 inhibition of ADP-induced washed platelet aggregation. 0.5ml aliquots of washed platelets were pre-incubated with $3x10^{-6}$M papaverine at 37°C for 30 seconds prior to the addition of EET. Following a 30 second pre-incubation with a sub-maximal dose of EET or PGI_2 the platelets were aggregated with ADP. Inhibitory effects of all four EETs and PGI_2 were augmented in the presence of papaverine. Mean percentage increases in inhibition were 31.5 $\pm$ 1.3 for 5(6)-EET, 17.0 $\pm$ 1.1 for 8(9)-EET, 43.3 $\pm$ 1.7 for 11(12)-EET, 24.3 $\pm$ 0.6 for 14(15)-EET and 32.8 $\pm$ 0.9 for PGI_2. Values are expressed as a mean $\pm$ SEM (n=4).

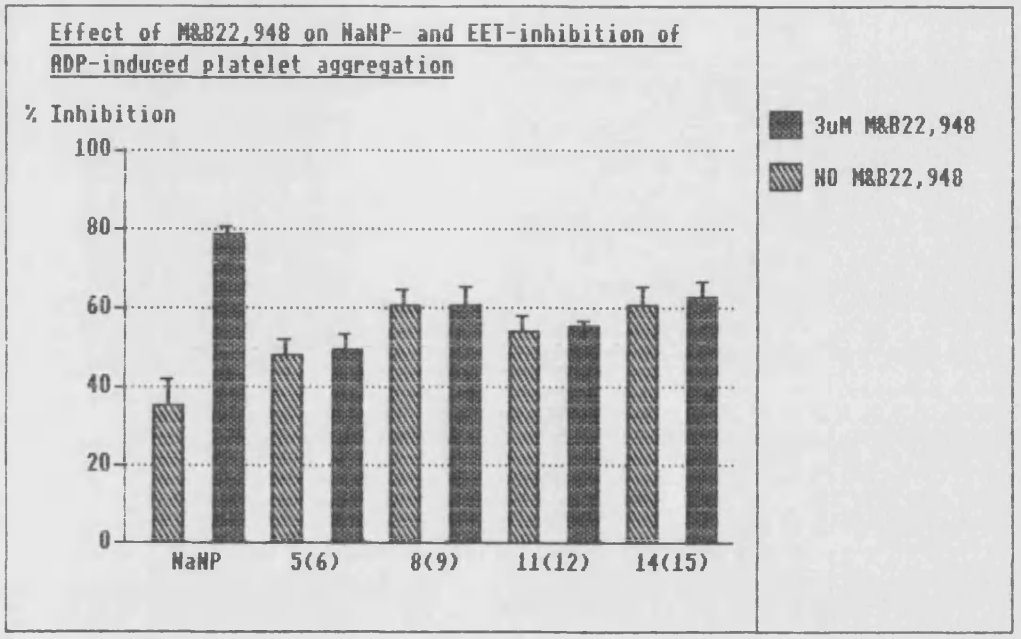


FIGURE 4.15

Effect of $3x10^{-6}$M M&B 22,948 on submaximal EET and NaNP inhibition of ADP-induced washed platelet aggregation. 0.5ml aliquots of washed platelets were pre-incubated with $3x10^{-6}$M M&B 22,948 at 37°C for 30 seconds prior to the addition of EET. Following a 30 second pre-incubation with a sub-maximal dose of EET or NaNP the platelets were aggregated with ADP. The inhibitory effects of NaNP were augmented in the presence of M&B 22,948 whereas the EET inhibition was unaltered. Mean percentage increases in inhibition were 1.3 $\pm$ 1.0 for 5(6)-EET, 0.5 $\pm$ 1.6 for 8(9)-EET, 1.3 $\pm$ 1.2 for 11(12)-EET, 2.0 $\pm$ 1.0 for 14(15)-EET and 43.0 $\pm$ 2.1 for NaNP. Values are expressed as a mean $\pm$ SEM (n=4).

4.4.5: Determination of EET Half-response Time

Pre-incubation of PGI_2 with buffer solution at 37°C demonstrated a biological half-response time of 52.0 ± 4.9 seconds (n=4) (Fig. 4.16). All anti-aggregatory activity was lost after 180 seconds pre-incubation. Utilising the data from both the PGI_2 dose-response and response decay curves an estimated half-life for PGI_2 of 31 seconds was obtained (range 26-38 seconds).

Application of the same technique to the EETs demonstrated half-response times of 6.3 ± 1.3 minutes for 5(6)-EET; 6.6 ± 1.2 minutes for 8(9)-EET; 6.2 ± 0.9 minutes for 11(12)-EET and 8.1 ± 1.1 minutes for 14(15)-EET (all n=4) (Fig 4.17). The anti-aggregatory activity of the EETs was not totally abolished through pre-incubation. The minimal anti-aggregatory activity observed for 5(6)-EET was 38.0 ± 3.3% (as a percentage of maximum aggregation), whilst that for the 8(9)-, 11(12)- and 14(15)-EETs was 29.8 ± 2.1%, 27.8 ± 2.1% and 28.5 ± 1.0% respectively. In order to obtain an indication of EET-breakdown, the decay profiles were adjusted so that the minimal response was taken as zero (Fig. 4.18). Accordingly adjusted half-response times of 2.6 ± 0.3, 3.5 ± 0.5, 3.8 ± 0.5 and 4.5 ± 0.4 minutes for 5(6)-, 8(9)-, 11(12)- and 14(15)-EET respectively were thus obtained. The approximate 'half-lives' of the 5(6)-, 8(9)-, 11(12)- and 14(15)-isomers were calculated as 70, 153, 147 and 230 seconds respectively (ranges: 39-103s,

110-197s, 101-195s and 137-324s respectively).

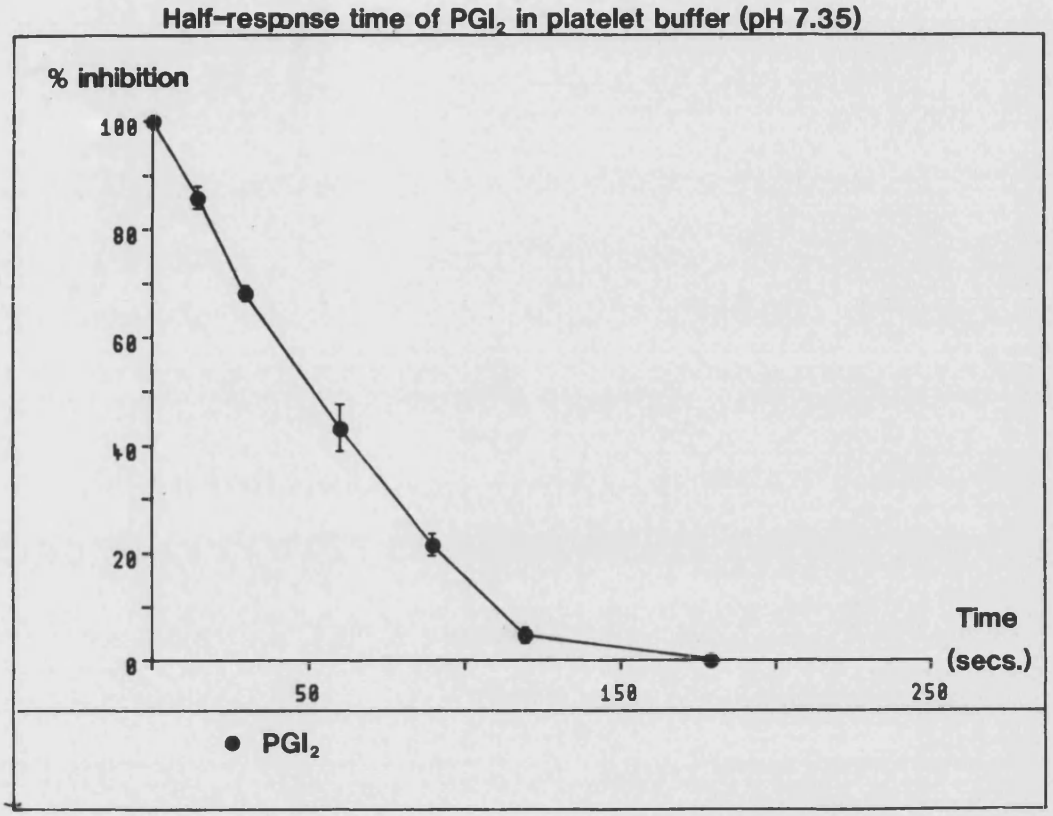


FIGURE 4.16

Decay profile of PGI_2 anti-aggregatory activity in platelet buffer. A maximal inhibitory dose of PGI_2 was pre-incubated in 0.1ml of platelet buffer at 37°C for a pre-determined period of time before the addition of 0.4ml platelet suspension. 30 seconds later a maximal aggregatory dose of ADP was added to the platelet suspension. All anti-aggregatory activity to PGI_2 was lost after 3 minutes pre-incubation with buffer. The estimated half-life was 31 seconds. Values are expressed as a mean $\pm$ SEM (n=4).

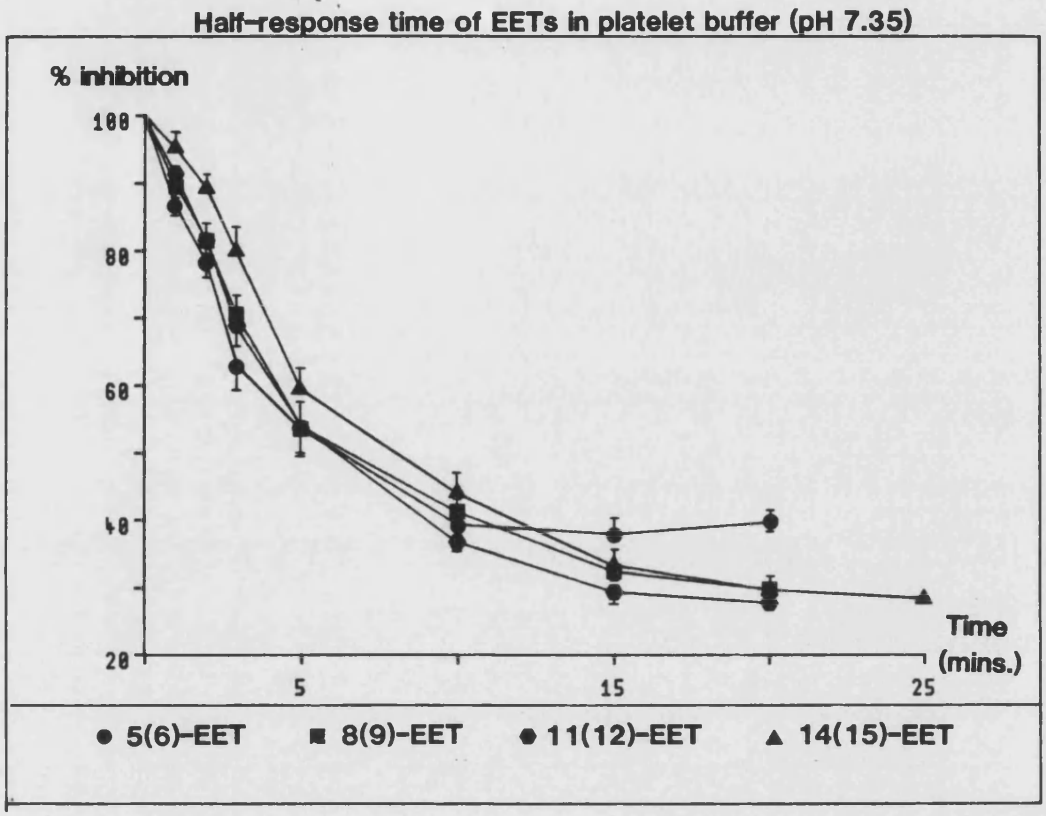


FIGURE 4.17

Decay profile of the EET inhibitory activities in platelet buffer. A maximal inhibitory dose of EET was pre-incubated in 0.1ml of platelet buffer at 37°C for a pre-determined period of time before the addition of 0.4ml platelet suspension. 30 seconds later a maximal aggregatory dose of ADP was added to the platelet suspension. 60% of the anti-aggregatory activity to 5(6)-EET and 70% to 8(9)-, 11(12)- and 14(15)-EET remained after 20 minutes pre-incubation with platelet buffer. To obtain an approximation of half-life the minimal inhibitory activity was adjusted to 100%. Values are expressed as a mean ± SEM (n=4).

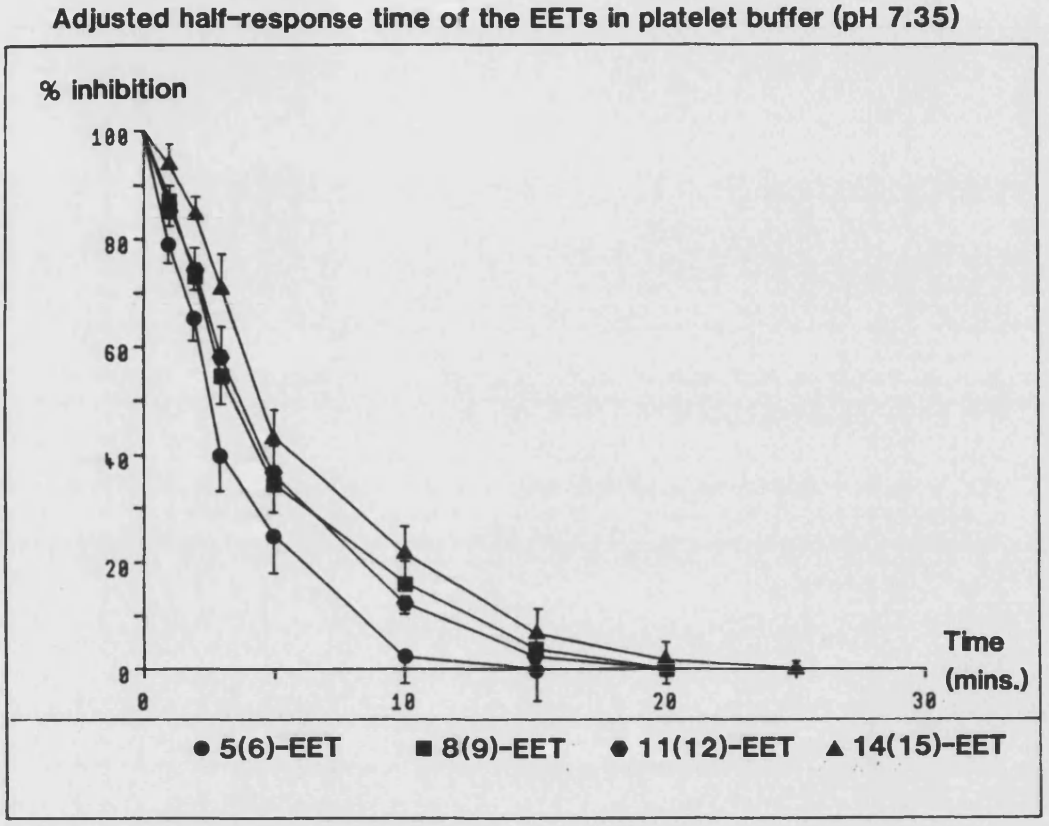


FIGURE 4.18

Adjusted decay profile of the EET inhibitory activity in platelet buffer. Approximate 'half-lives' for the EETs were 70 seconds for 5(6)-EET, 153 seconds for 8(9)-EET, 147 seconds for 11(12)-EET and 230 seconds for 14(15)-EET. Values are expressed as a mean ± SEM (n=4).

4.5: DISCUSSION

4.5.1: Choice of Platelet Preparation

The use of washed platelets had several advantages over PRP. Due to the increased sensitivity of the washed platelets, compared to PRP, smaller quantities of drugs and synthetic compounds were required to induce responses, so reducing the demand on resources. This was undoubtedly true for the epoxyeicosatrienoic acids considering the arduous preparation and low resultant yields. Also by excluding plasma proteins from the platelet suspension, thrombin could be readily utilised as an aggregatory agent. Also, by avoiding the use of PGI_2 in platelet preparation, the isolated platelet suspension could be used immediately rather than having to stand for several hours for the PGI_2 activity to decline.

The 10-fold increase in responsiveness to ADP of the platelets following resuspension in Ca^{2+}-free HEPES-Tyrodes was probably due to the absence of plasma proteins and enzymes found in PRP which could bind or metabolise the pro- and anti-aggregatory agents used. In the cases of the EETs, the apparently reduced potency may be due to epoxide hydrolase in PRP preparations (Chacos et al., 1983).

4.5.2: Half-response Time Determination

The responsive nature of washed platelets to both agonists and inhibitors of platelet function highlighted their suitability in the determination of biological stability of the EETs in aqueous conditions. Authentication of the technique, using PGI_2 as test compound, indicated it to be accurate giving an aqueous half-life for PGI_2 of 31 seconds. However, in order to obtain a viable estimation of half-life any break-down products must be physiologically inactive, indicated by the decay profile falling to zero. Unfortunately, the analysis of EET-stability was complicated by the anti-aggregatory activity not falling to zero. It must be stressed therefore that the instability profile so obtained for the EETs can only be used as a guidance to their stability in aqueous conditions. Even so, it is still reasonable to conclude that the life-time of these compounds in physiological fluids is relatively short and so could probably act as local mediators of physiological responses.

Of interest is the apparent anti-aggregatory activity of the degradation products, presumably the DHETs and the d5-lactone. Previous publications have indicated some biological activity for these compounds in other preparations (Capdevila et al., 1983).

4.5.3: Effect of Indomethacin and NDGA on EET-inhibition of Washed Platelet Aggregation

Fitzpatrick et al. (1986, 1987) demonstrated micromolar concentrations of 8(9)-, 11(12)- and 14(15)-EETs to be anti-aggregatory in human platelets activated by AA. The findings reported here endorse and extend these findings to rat platelets stimulated by ADP or thrombin. Furthermore, the 5(6)-isomer also proved to be anti-aggregatory, this property not being reported by Fitzpatrick et al. (1986, 1987).

The greater potency of 5(6)-EET does not appear to be due to it's further metabolism by cyclo-oxygenase despite being a substrate for this enzyme (Oliw, 1984). Blockade of the cyclo-oxygenase pathway by indomethacin augmented EET-inhibition by all four isomers. If an anti-aggregatory epoxyprostaglandin is responsible for the greater potency of the 5(6)-isomer, then an attenuation of 5(6)-EET-induced inhibition of aggregation would have been observed in the presence of indomethacin. However the failure to completely block AA-induced aggregation by the concentration of indomethacin used would suggest only a partial blockade of cyclo-oxgenase activity was achieved. Increasing the pre-incubation period with indomethacin may have improved the blockade of AA-induced aggregation. Nevertheless, a reduction in 5(6)-EET potency following a reduction in cyclo-oxygenase activity would have been observed whereas

augmentation resulted.

The absence of a significant reduction in inhibitory activity following NDGA- and indomethacin-induced blockade would suggest that further metabolism via the cyclo-oxygenase or lipoxygenase pathways is not responsible for the greater potency of 5(6)-EET. Moreover the other three EETs also will not act through cyclo-oxygenase-dependent mechanisms as they are not substrates. The anti-aggregatory activity of these EETs was indeed found to be unaffected by indomethacin. Lipoxygenase involvement in 8(9)-, 11(12)- and 14(15)-EET inhibition of platelet aggregation is also ruled out as the inhibition was not attenuated by NDGA. Thus the biological activity of the EETs in platelets appears to be totally independent of these two pathways.

4.5.4: Effect of M&B 22,948 on EET-inhibition of Washed Platelet Aggregation

Examination of the roles of cyclic nucleotides in the EET-inhibition of platelet aggregation indicated the participation of cAMP but not cGMP. Pre-incubation of the platelets with M&B22,948, a specific inhibitor of cGMP-phosphodiesterase (Weishaar et al., 1986), had no significant effect on EET-inhibition of ADP-induced aggregation, whilst augmenting inhibition by NaNP. Pre-incubation with Papaverine, a non-selective phosphodiesterase inhibitor (Kramer & Wells, 1979;

Weishaar et al., 1986), augmented the inhibitory effects of both the EETs and PGI_2. However, an increased inhibition of platelet aggregation following cAMP-phosphodiesterase inhibition is not surprising considering the central role of cAMP in suppressing platelet aggregation (Haslam et al., 1978). Furthermore, the involvement of cAMP in EET-inhibition of platelet aggregation is disputed by their inability to increase cAMP levels in human platelets (Fitzpatrick et al., 1986). The findings that neither cyclo-oxygenase or lipoxygenase nor cyclic nucleotides are essential to the inhibitory actions of the EETs suggest an independent mechanism is operative.

4.5.5: Other Mechanisms to be Considered

Investigations into the biological activity of the EETs on human washed platelets by Fitzpatrick et al. (1986) uncovered an anomaly in their apparent mechanism of action when compared with other anti-aggregatory compounds. Normally, the addition of an anti-aggregatory agent induces a reduction in the platelet TXB_2 level brought about by an increase in cAMP levels (Malmsten et al., 1976; Fitzpatrick & Gorman 1979). Such a response was not observed with EET-induced anti-aggregatory effects. However, the phosphorylation of a 40-kDa platelet protein, another indicator of platelet activation (Lyons et al., 1975), was reported to behave

as expected on exposure to the anti-aggregatory EETs (Fitzpatrick et al., 1986). This would suggest a mechanism by which the EETs could act distally to thromboxane synthesis, perhaps as an antagonist at the TX receptor. Such receptor inhibition has already been described for other polyunsaturated fatty acids. Antagonism of TX receptors would block the positive feed-back loop of TXA_2 production and halt the propagation of platelet aggregation. The changes in phosphorylation of a 40-kDa protein following EET-inhibition of platelet aggregation would suggest its involvement distal to TX production. Closer scrutiny of this divergence in effects may reveal a mechanism of action universal to EET-induced responses.

The central role of cytosolic calcium in platelet activation is well documented (Seiss, 1989). Associated with platelet activation is an increase in free cytosolic calcium. Of interest therefore is the reported effects of the EETs on Ca^{2+} in anterior pituitary cells (Snyder et al., 1986), canine aortic smooth muscle and rat liver microsomes (Kutsky et al, 1983). Exposure of anterior pituitary cells to micromolar concentrations of 5(6)-EET resulted in an increase in cytosolic calcium levels. Similarly, exposure to 14(15)-EET increased Ca^{2+} release and decreased active Ca^{2+} uptake in canine aortic and rat liver microsomes. Such EET-induced effects would appear to contrast with their inhibitory effects in human and

rat platelets, where an increase in cytosolic Ca^{2+} levels would initiate aggregation. These discrepancies suggest primary differences in calcium release mechanisms between platelets and other tissues. Furthermore, it may not be valid to compare the actions of the EETs in an intact physiological system such as platelets and isolated microsomal fractions separated from cellular influence. Alternatively, any calcium liberation which may be induced in platelets could be masked or overcome by the predominant mechanism responsible for EET anti-aggregatory activity.

Further investigation into EET-inhibition of platelet aggregation is essential if we are to understand their inhibitory nature and resolve some of the apparent discrepancies between known actions.

CHAPTER 5: MICROSOMAL SYNTHESIS OF THE EETs

5.1: INTRODUCTION

The synthesis of all four EETs and their corresponding diols has been demonstrated utilising microsomes from rabbit and rat liver (Oliw et al., 1982; Chacos et al., 1982). Furthermore 11,12- and 14,15-DHETs have been isolated from rabbit liver and renal cortex (Oliw and Moldeus, 1982), 8(9)- and 14(15)-EETs being isolated from rabbit whole kidney (Falck et al., 1987). However, although cytochrome P_{450} monooxygenase has been located in many extrahepatic sites including the vasculature (Juchau et al., 1976; Baird et al., 1980; Dees et al., 1980; Abraham et al., 1985; Serabjit-Singh et al., 1986), the synthesis of EETs by these tissues has not been observed. Due to the lability of the EETs, demonstration of their syntheses at or near their site of action is necessary before concluding any physiological relevance in that tissue. For this reason attempts to demonstrate aortic microsomal synthesis of the EETs were pursued.

Induction and depletion of cytochrome P_{450} has been demonstrated to affect some endothelium-dependent relaxations. Mono-oxygenase induction by 3-methyl-cholanthrene and β-naphthoflavone caused an increase in AA-induced relaxation in rabbit pulmonary artery (Pinto et al., 1986) and canine coronary artery (Pinto et al., 1987). Depletion of cytochrome P_{450} by cobalt chloride

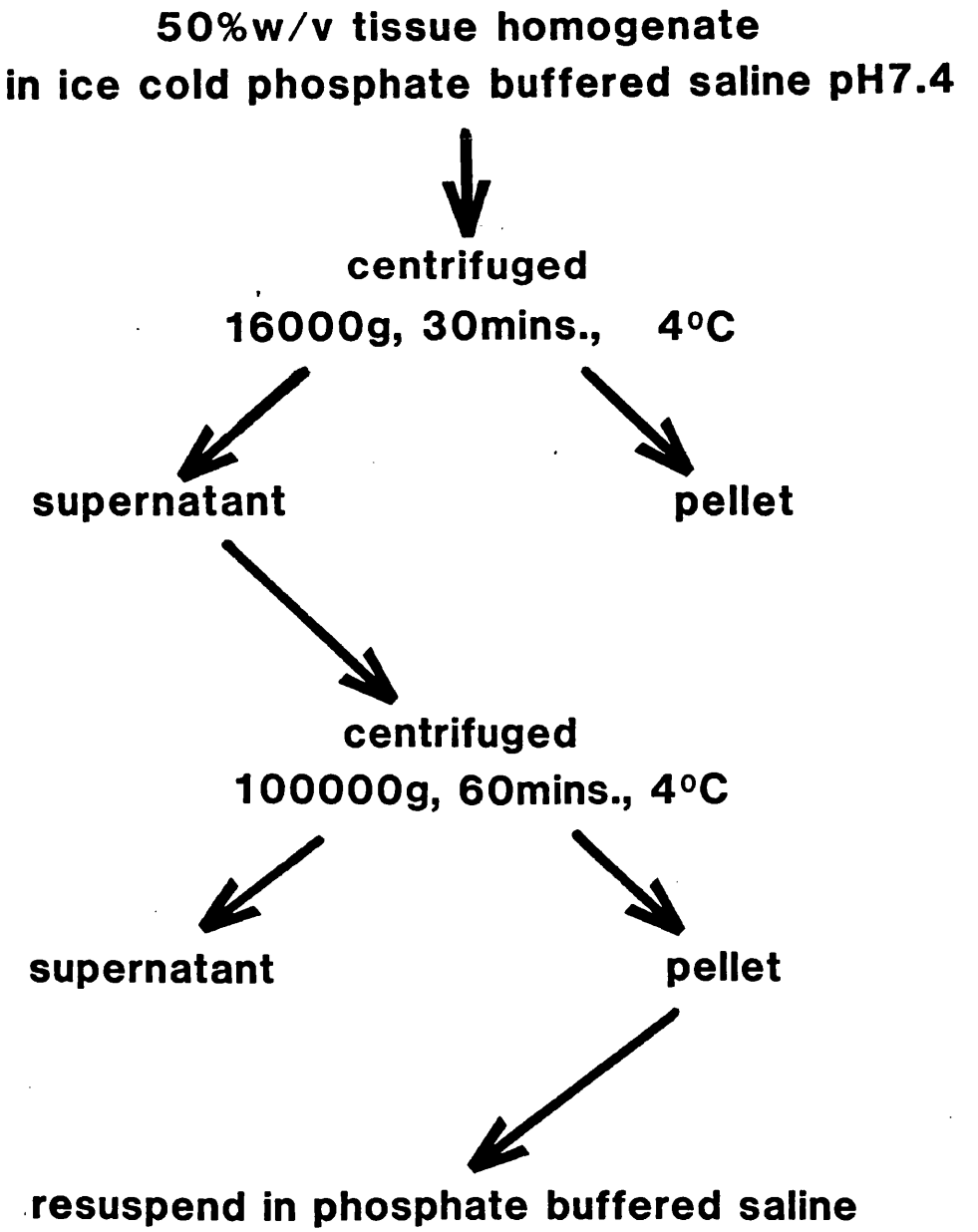
reduced AA-induced relaxations in both tissues. Furthermore, endothelium-dependent ACh-induced relaxations in the rat superior mesenteric bed were augmented by pre-treatment of the rats with phenobarbitone (Pb) (Randall & Hiley, 1988), the concentration of cytochrome P_{450} being elevated following Pb treatment.

5.2: METHODS

5.2.1: Preparation, Assessment and Incubation of Microsomes from Rat Aorta

The tissue to be examined for EET synthesis was removed from the animal and immediately placed in ice-cold phosphate buffered saline at pH 7.4. All traces of fat and connective tissue were removed and a 50% w/v tissue homogenate prepared in cold phosphate-buffered saline. The homogenate was centrifuged at 16,000g for 30 minutes to remove unbroken cells, nuclear, mitochondrial and lysosomal debris. The supernatant was re-centrifuged at 100,000g for 60 minutes to sediment the microsomal protein. The pellet was re-suspended in fresh cold phosphate-buffered saline. The entire production of the microsomal suspension was carried out on ice or at a controlled temperature of 4°C.

The protein content of the microsomal suspension was assessed using the G-250 dye binding method. In brief, a range of protein solutions of known concentration between 0.1mg/ml and 1mg/ml were prepared from bovine serum albumin (BSA). To 0.1ml of each solution was added 5ml of fresh G-250 reagent and after 2 minutes the absorbance measured at 595nm. A standard plot was constructed from the BSA data. 10 fold serial dilutions of an aliquot of the microsomal suspension were prepared and processed as above. The protein content of the suspension could then


50%w/v tissue homogenate
in ice cold phosphate buffered saline pH7.4
centrifuged
16000g, 30mins., 4°C
supernatant
pellet
centrifuged
100000g, 60mins., 4°C
supernatant
pellet
resuspend in phosphate buffered saline

be determined by extrapolation from the standard plot.

Aliquots of the protein suspension were incubated with 25ug/ml 1-C^{14} AA in the presence of 1mM NADPH for 60 minutes at 37°C. Any products and residual AA were extracted using 0.1ml ethylacetate after acidification of the microsomal suspension with 0.05ml 0.1M hydrochloric acid. The extraction was repeated twice and the organic phases pooled.

5.2.2: Effect of P_{450}-Induction on AA Metabolism

Three groups of five male Wistar rats, weight 330g to 370g, were given either B-naphthoflavone (100mg/Kg/day i.p.), phenobarbitone (75mg/Kg/day i.p.) or araclor 1254 (50mg/Kg/day i.p.) on three consecutive days. In all cases the drug was suspended in 2ml olive oil. A fourth, control, group was injected i.p. with 2ml olive oil. On the fourth day the aortas from all five rats in each group were removed, pooled and microsomal suspensions prepared as described above. 0.02mg of protein fortified with 1mM NADPH from each group was incubated with radiolabelled AA. The products were separated by TLC to 11cm from the origin (solvent 35%v/v ethylacetate in hexane, containing 0.1% acetic acid), and an autoradiograph developed to visualise the metabolites formed. The relative product synthesis was estimated using an LKB 2202 Ultrascan Laser Densitometer to analyse the autoradiograph.

5.2.3: AA Metabolism by Rat Aortic Microsomes

20 male Wistar rats where pre-treated with 75mg/Kg phenobarbitone on three consecutive days to induce cytochrome P_{450} monooxygenase. On the fourth day the rats were killed and the thoracic aortae were removed. A microsomal suspension was prepared as described in section 5.2.1 and 0.92mg microsomal protein incubated in the presence and absence of 1mM NADPH as previously described. Following extraction of the products and residual AA the samples were methylated and silylated. Incubation products were separated using gas chromatography as described in chapter 2.

5.3: RESULTS

5.3.1: Effect of P_{450}-Induction on AA Metabolism

Incubation of NADPH-fortified aortic microsomes (0.02mg protein) from control rats, with 1-^{14}C radio-labelled AA, gave products with Rf values of 0.44, 0.29, 0.22 and 0.15. Incubation of NADPH-fortified microsomes (0.02mg protein) from rats treated with inducing agents gave products with similar Rf values to those obtained from untreated rats. In addition a product with an Rf value of 0.36 was produced by NADPH-fortified aortic microsomes from rats pre-treated with phenobarbitone. This band was not produced in any significant quantities by microsomes from rats pretreated with β-naphthoflavone, araclor 1254 or by the control group. Furthermore the total product formation, as determined by laser densitometry, by microsomes from phenobarbitone treated rats (area = 6.1%) was greater than that produced in the other incubations (area 3.3-4.1%). The band at Rf 0.44 also appeared in the negative control AA sample subjected to TLC (Figure 5.1). Analysis of the autoradiograph using laser densitometry clearly indicated the presence of an additional metabolite (Fig. 5.2). The relative intensities of the bands are summarised in table 5.1 (page 177).

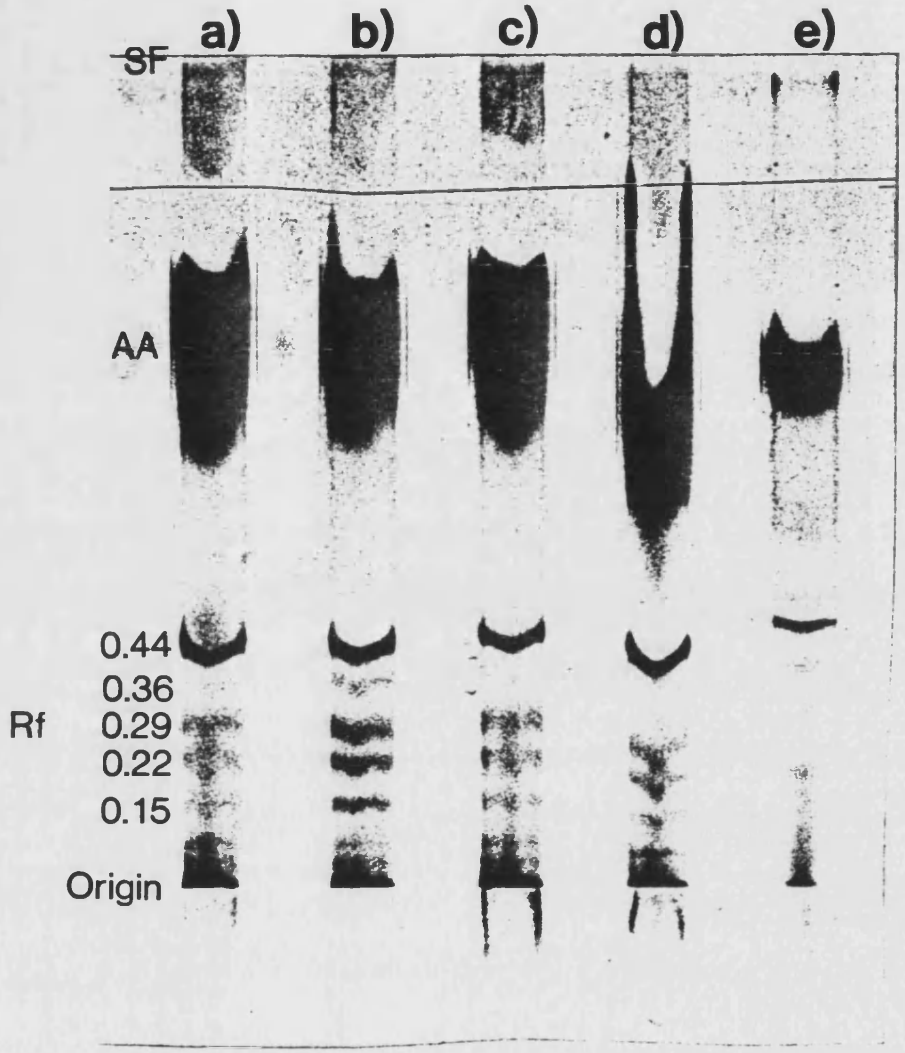
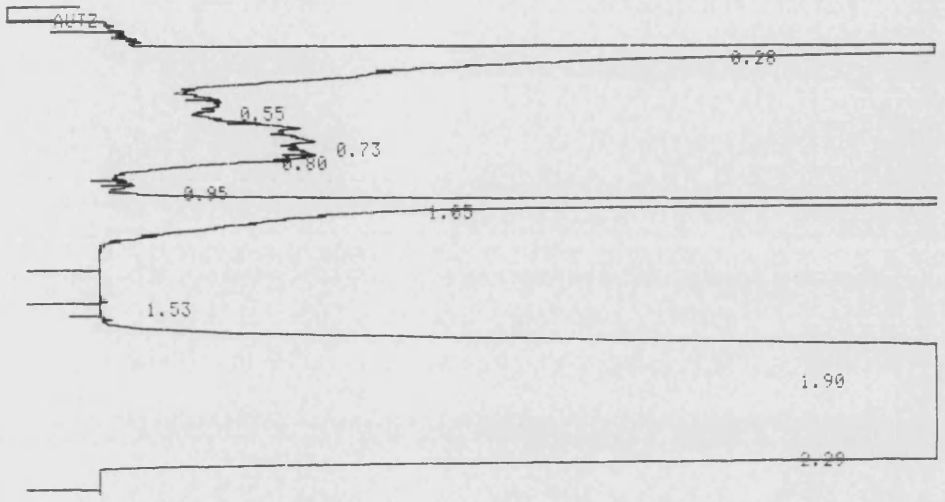


FIGURE 5.1
Autoradiograph following TLC separation showing the products of AA metabolism by aortic microsomes from a) araclor 1254 treated rats, b) phenobarbitone treated rats, c) b-naphthoflavone treated rats and d) control rats. Lane e) shows AA breakdown in the incubation medium.

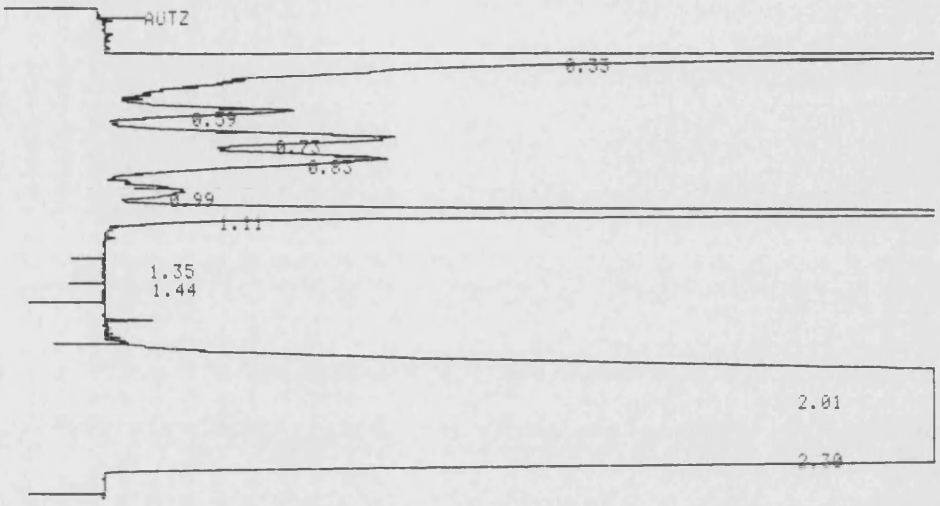


RUN TIME 2.47

DEFAULT 0
NORMALIZATION METHOD USING AREA

TIME	HEIGHT	AREA		%AREA
0.28	860255	10559882	P	11.9163070
0.55	29353	311915	SP	0.3519807
0.73	104504	1507776	SP	1.7014510
0.80	115971	1079910	S	1.2186253
0.95	8919	38858	S	0.0438493
1.05	926513	6242907		7.0448135
1.53	4006	5084	P	0.0057370
1.90	987590	41567366	P	46.9067280
2.29	987588	27303367		30.8105060
TOTAL		88617065		100.0000000

FIGURE 5.2a
Laser densitometer analysis of TLC autoradiograph from araclor pretreated rat aortic NADPH-fortified microsomes incubated with 1-^{14}C AA.



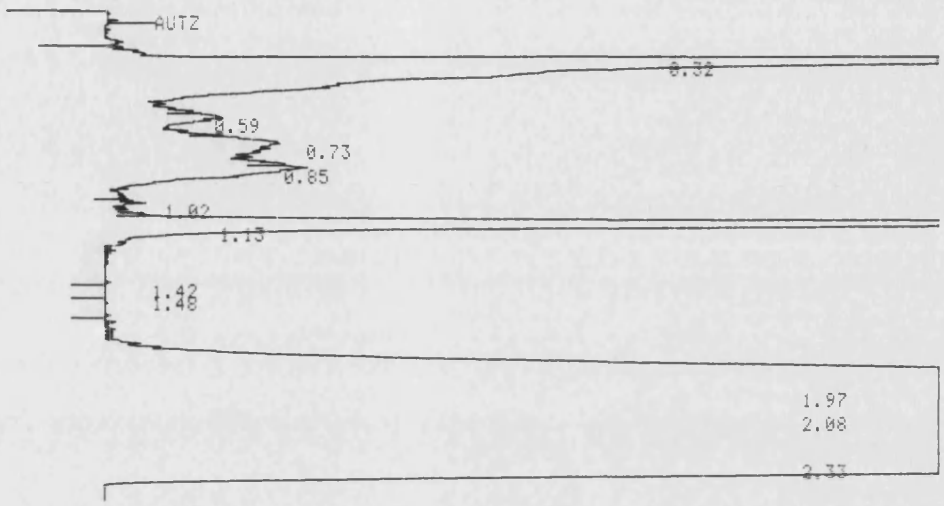
RUN TIME 2.49

DEFAULT 0
NORMALIZATION METHOD USING AREA

TIME	HEIGHT	AREA		%AREA
0.33	752864	6004319	P	8.4103263
0.59	117162	716196	P	1.0031848
0.73	178043	1591784	P	2.2296321
0.83	180653	1659656	P	2.3247013
0.99	49841	410561	P	0.5750780
1.11	840378	4244607		5.9454752
1.35	147	356	P	0.0004986
1.44	1	146		0.0002045
2.01	988075	37810757	P	52.9620100
2.30	988066	18953842		26.5488880
TOTAL		71392224		100.0000000

FIGURE 5.2b

Laser densitometer analysis of TLC autoradiograph from phenobarbitone pretreated rat aortic NADPH-fortified microsomes incubated with 1-^{14}C AA. The peak at time 0.99 represents the additional product at Rf 0.36 produced by phenobarbitone induced microsomes.

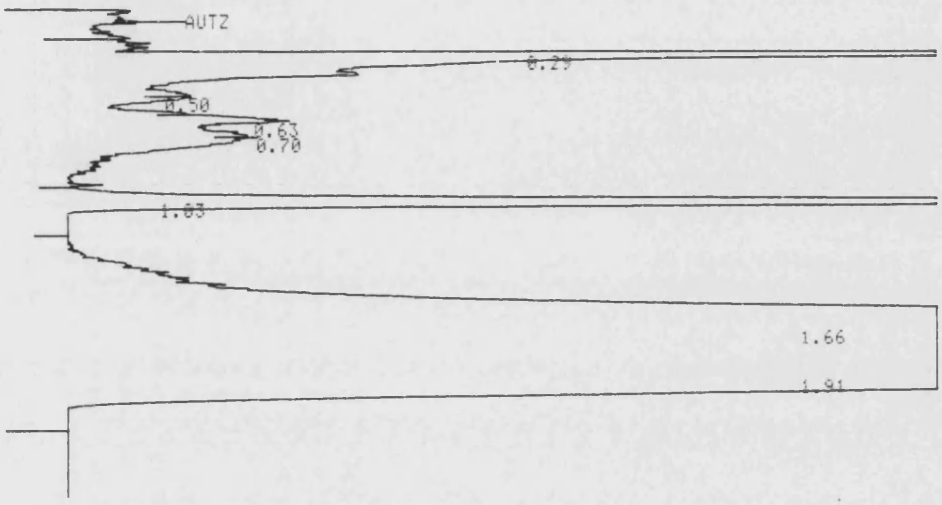


RUN TIME 2.48

DEFAULT 0
NORMALIZATION METHOD USING AREA

TIME	HEIGHT	AREA		%AREA
0.32	882715	9209704	P	11.3586706
0.59	47490	363722	SP	0.4485918
0.73	88815	1186732	SP	1.4636407
0.85	109940	1201809	S	1.4822357
1.02	10808	22421	S	0.0276526
1.13	818898	4548104		5.6093459
1.42	1	1251	P	0.0015429
1.48	1	1069	P	0.0013184
1.97	988093	26874682	P	33.1455450
2.08	988102	20749413	P	25.5910230
2.33	988109	16921919		20.8704320
TOTAL		81080826		100.0000000

FIGURE 5.2c
Laser densitometer analysis of TLC autoradiograph from b-naphthoflavone pretreated rat aortic NADPH-fortified microsomes incubated with 1-^{14}C AA.

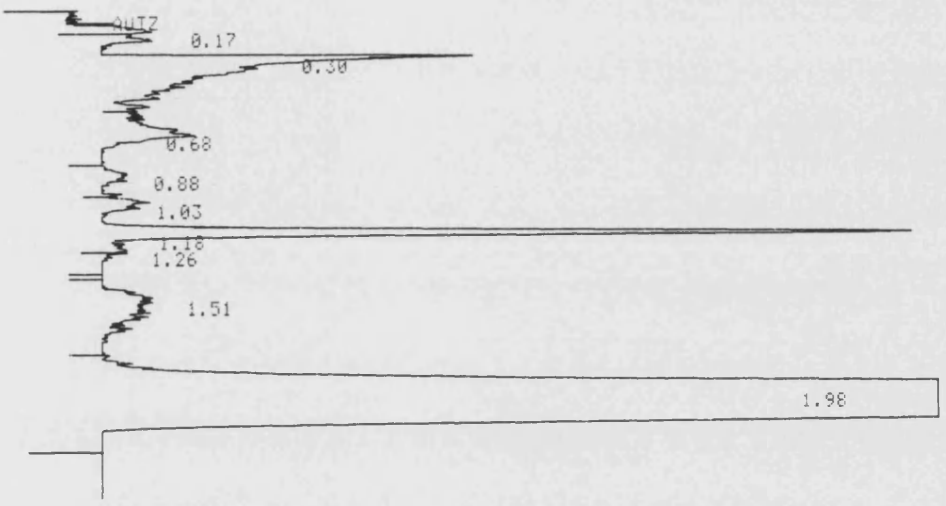


RUN TIME 2.46

DEFAULT 0
NORMALIZATION METHOD USING AREA

TIME	HEIGHT	AREA		%AREA
0.29	748379	6138537	P	9.2491346
0.50	36190	207015	S	0.3119162
0.63	128474	1078507	P	1.6250218
0.70	114463	1421895		2.1424157
1.03	987534	4899109		7.3816479
1.66	988271	36754704	P	55.3795150
1.91	988266	15869004		23.9103470
TOTAL		66368771		100.0000000

FIGURE 5.2d
Laser densitometer analysis of TLC autoradiograph from control pretreated rat aortic NADPH-fortified microsomes incubated with 1-^{14}C AA.



RUN TIME 2.47

DEFAULT 0
NORMALIZATION METHOD USING AREA

TIME	HEIGHT	AREA		%AREA
0.17	29376	243064		0.8153787
0.30	188308	2430498	P	8.1533112
0.68	48559	529027	S	1.7746658
0.88	15027	113429	P	0.3805071
1.03	20382	186577	P	0.6258883
1.18	464178	2053181		6.8875694
1.26	8196	10716	S	0.0359477
1.51	27987	557924	P	1.8716032
1.98	988377	23685534		79.4551280
TOTAL		29809950		100.0000000

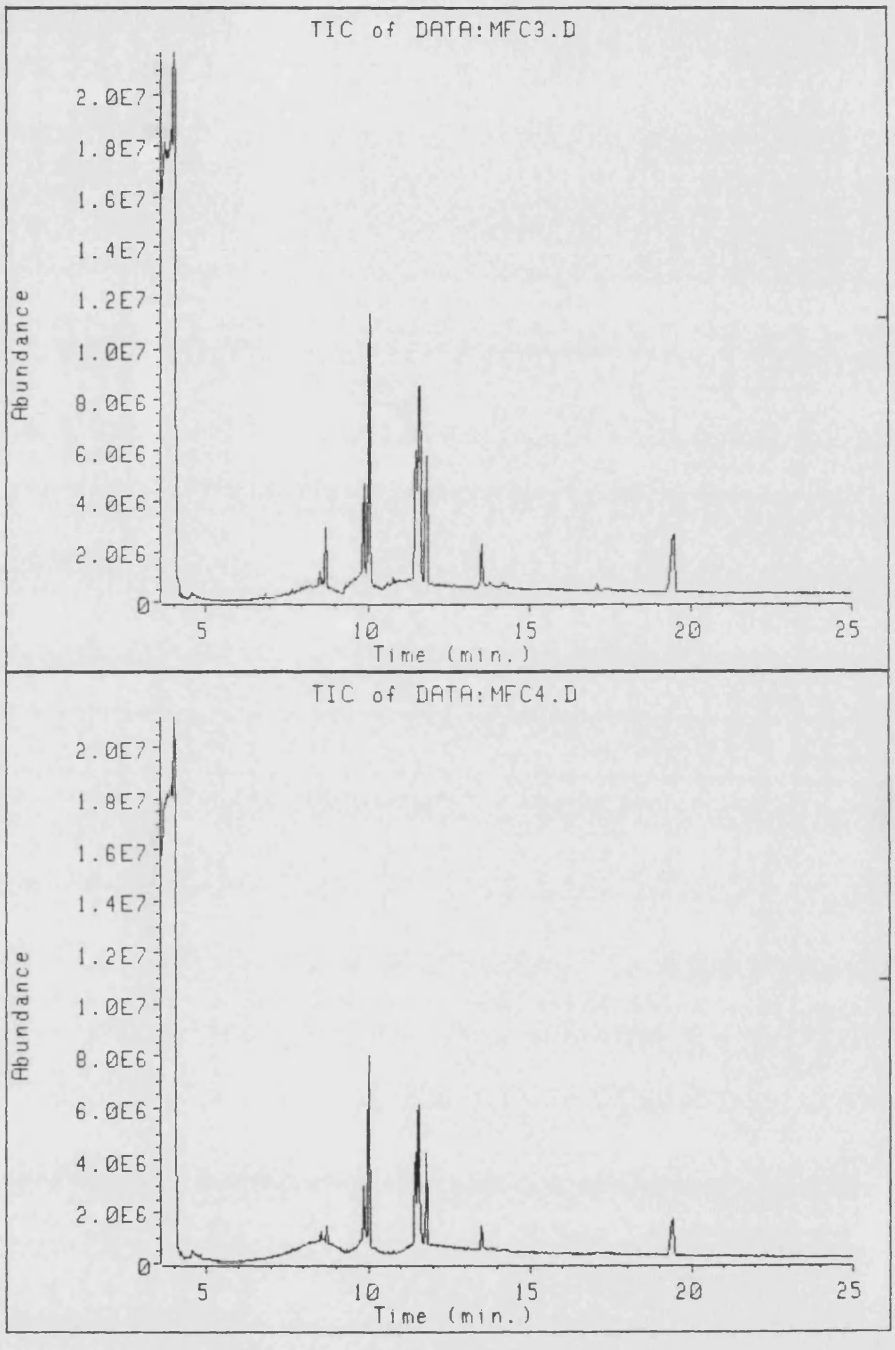
FIGURE 5.2e
Laser densitometer analysis of TLC autoradiograph from AA breakdown in the incubation medium over the time period of the experiment.

Table 5.1
Rf values and band intensities from autoradiograph assessed by laser densitometry of the products from AA-incubation with rat aortic microsomes taken from a control group, and groups pretreated with araclor 1254, phenobarbitone and b-naphthoflavone (n=1).

araclor		PB		b-NF		CONTROL	
Rf	%Area	Rf	%Area	Rf	%Area	Rf	%Area
AA	77.72	AA	78.51	AA	79.61	AA	79.29
0.44	7.04	0.44	5.95	0.44	5.61	0.44	7.38
		0.36	0.58				
0.29	1.21	0.29	2.32	0.29	1.48	0.29	2.14
0.22	1.70	0.22	2.23	0.22	1.46	0.22	1.62
0.15	0.35	0.15	1.00	0.15	0.45	0.15	0.31

5.3.2: AA Metabolism in Rat Aorta

The spectrum of AA-metabolites following incubation with rat aortic microsomes in the presence and absence of 1mM NADPH was qualitatively similar. However the quantity of a group of products which eluted between 8 and 9 minutes during gas chromatography (on an OV-1, 12m capillary column) was greatly increased relative to the other metabolites by the addition of 1mM NADPH to the incubation medium (Fig. 5.3). The identities of these compounds was not ascertained.



FUGURE 5.3
GC trace of phenobarbitone-induced rat aortic microsomal metabolites of AA in a) the presence of 1mM NADPH and b) absence of NADPH.

5.3: DISCUSSION

Preliminary investigations into AA-metabolism by rat aortic microsomes demonstrated a change in the profile of products formed by both Pb-induction and NADPH-fortification of microsomal suspension. Pre-treatment of the test animals with phenobarbitone has been shown to preferentially increase the amounts of EETs synthesised (Oliw et al., 1982), whilst NADPH is known to be essential for cytochrome P_{450} activity. Although the identities of the metabolites enhanced by Pb-treatment and NADPH-fortification were not determined, the results would suggest that phenobarbitone-inducible cytochrome P_{450} activity can be found in rat aorta. Furthermore, although the identities of the AA-metabolites increased by the presence of NADPH and eluting from the GC-column between 8 and 9 minutes were not identified, it is interesting to note that the methylated EETs also eluted at this time. In order to reach a firm conclusion that the EETs are produced in aortic tissue will require the isolation and positive GC-MS identification of either the EETs or their respective DHETs against standards. Chemical synthesis of the DHETs and d5-lactone can be achieved from the EETs as described by Oliw and Moldeus (1982).

To demonstrate the biological importance of these compounds in the tissues tested requires evidence of their *in vivo* synthesis. Unfortunately time prevented

anything more than some basic preliminary investigations into this important topic. A detailed examination of microsomal AA-metabolites, especially from phenobarbitone pre-treated animals, may reveal the DHETs, or in the presence of an epoxide hydrolase inhibitor, the EETs.

CHAPTER 6: GENERAL DISCUSSION AND OVERVIEW

Biological responses to the epoxyeicosatrienoic acids appear to be as diverse as those observed for the other eicosanoids. This report describes the relaxant properties in two separate smooth muscle preparations as well as anti-aggregatory actions to all four isomers.

In addition to the intrinsic relaxant actions of the EETs, the 5(6)-EET may act via the cyclo-oxygenase pathway. This additional feature of the 5(6)-isomer is due to its residual 8,11,14-*cis*-triene configuration following cytochrome P_{450} metabolism of AA. The results provided here together with a report by Carroll et al (1988) suggest that epoxyprostaglandins are responsible for at least some of the activity induced by 5(6)-EET. It is therefore prudent to consider such cyclo-oxygenase metabolism when investigating the biological actions of this compound. The work of Oliw (1984) identified 5-hydroxy-$PGI_{1\alpha}$, 5-hydroxy-$PGI_{1\beta}$, 5(6)-epoxy-$PGF_{1\alpha}$, 5(6)-epoxy-PGE_1, 5,6-dihydroxy-PGE_1 and the epoxy-PG endoperoxides as cyclo-oxygenase metabolites of 5(6)-EET. However the conversion of the 5(6)-epoxyendoperoxides by thromboxane synthetase has not been demonstrated, such possible production of an epoxythromboxane warranting investigation. Furthermore secondary conversion of the EETs by the lipoxygenase pathway may also have implications in the actions of the EETs, although NDGA had no effect on the EET-induced responses reported here.

Glutathione conjugation of the EETs has been described, the 14(15)-EET being the preferred substrate of the four EETs (Spearman et al., 1985).

Although many interesting results have been obtained further investigations are warranted particularly with respect to cyclic nucleotides. The effects of the cGMP-phosphodiesterase inhibitor, M&B 22,948, demonstrated the importance of cGMP in the relaxant actions of the less potent EETs but not in the anti-aggregatory actions of the EETs. In addition, experiments with specific cAMP-phosphodiesterase inhibitors may disclose cAMP-dependent effects to 5(6)-EET in smooth muscle. The synthesis of some 5(6)-epoxyeicosanoids, notably 5(6)-epoxy-PGE_1 and 5(6)-hydroxy-PGI_1s, and their subsequent bioassay would prove informative.

The reported increases in anterior pituitary cell cytosolic Ca^{2+} (Snyder et al. 1986) and Ca^{2+} release by liver and aortic microsomes (Kutsky et al. 1983) would appear to be at odds with the relaxant and anti-aggregatory activity demonstrated here and elsewhere (Carroll et al., 1987, 1988; Procter et al., 1987; Fitzpatrick et al., 1986, 1987). More information concerning the influence of the EETs on Ca^{2+} mobilisation may help unify these variant actions.

Increases in cytosolic calcium were observed in LHRH-induced LH release and EET-induced LH-release (Snyder et al., 1983; 1986). A role for calcium as a

second messenger in LHRH stimulation of the anterior pituitary rather than cAMP or cGMP has been suggested by Conn et al., (1981). However it could not be deduced whether LHRH or EET simply increased the permeability of the anterior pituitary cells to calcium, or induced its release from internal stores such as the endoplasmic reticulum.

The involvement of calcium as a second messenger rather than cAMP has also been implicated in the release of prolactin (Thorner et al., 1980). EET-induced mobilisation of calcium could account for the stimulated prolactin release from GH3 cells following EET exposure as described by Cashman et al. (1987).

Cytochrome P_{450} involvement has also been implicated in endothelium-dependent relaxations. AA-induced endothelium-dependent relaxations have been reported in canine coronary and superior mesenteric arteries (Furchgott, 1983; Pinto et al., 1987), rabbit pulmonary artery & thoracic aorta (Singer & Peach, 1983; Pinto et al., 1986) and rat thoracic aorta (Davies & Williams, 1984). Cytochrome P_{450} metabolites of AA have been proposed as the mediators of AA-induced endothelium-dependent relaxations (Pinto et al. 1986, 1987), cytochrome P_{450} activity having been demonstrated in various blood vessels of different species (Juchua et al., 1976; Baird et al., 1980; Dees et al., 1982). Cytochrome P_{450} activity has been detected both in the endothelium (Abraham et al., 1985) and in the smooth

muscle (Serabjit-Singh et al., 1985). Manipulations of the cytochrome P_{450}-dependent pathway were shown to alter the endothelium-dependent responses to AA. 3-Methyl-cholanthrene (3-MC) and β-naphthoflavone (β-NF) which induce cytochrome P_{450}, enhanced AA-induced relaxations whereas cobalt chloride ($CoCl_2$) which is known to deplete cytochrome P_{450} levels attenuated AA-induced responses (Pinto et al., 1986; 1987). ACh-induced endothelium-dependent relaxations have also been shown to be enhanced by inducing cytochrome P_{450} with phenobarbitone in test animals prior to use (Randall & Hiley, 1988). In addition, a direct role for cytochrome P_{450} in ACh-induced relaxations has also been proposed (Rubanyi and Vanhoutte, 1987).

Arachidonic acid is not the only unsaturated fatty acid (FA) capable of inducing endothelium-dependent relaxations. However the doses required of the other FAs is much greater than that of AA (Cherry et al., 1983; Furchgott, 1984). It is interesting to note though that the next most potent unsaturated FA, docosahexaenoic acid, is also a substrate for cytochrome P_{450} metabolism (Van Rollins et al., 1984) possibly indicating the importance of this pathway. Alternatively endothelium-dependent relaxations to other unsaturated FAs could be due to an increase in membrane fluidity (*see* reviews by Furchgott, 1983, 1984).

Over the last three years there have been two other reports of EET-induced biological activity and a further

observation of EET synthesis. 5(6)-, 11(12)- and 14(15)-EET and the DHETs have been demonstrated to inhibit forskolin- and vasopressin-stimulated osmotic water flow in the toad bladder (Schlondorff et al., 1987). The 5(6)- and 11(12)-isomers were roughly equipotent, 14(15)-EET being less so. Increased levels of cAMP in the bladder following vasopressin or forskolin stimulation were found to be attenuated by the EETs. This attenuation of cAMP formation by the EETs was parallelled by their ability to decrease water flow across the bladder. All the effects observed appeared to be independent of PG production. Of interest was the ability of 11(12)-EET to inhibit cAMP or 8-BrcAMP induced water flow, indicating an additional mechanism of action for this isomer independent of cAMP. The activity of the 8(9)-isomer was not investigated.

Secondly, incubation of arachidonic acid with microsomes of bovine adrenal fasciculata cells, in the presence of NADPH, resulted in the production of DHETs and w-hydroxylation products (Nishimura et al., 1989). Althought the EETs were not identified in the incubate, the appearance of their hydrolysis products suggested they were synthesised (Oliw et al., 1982). However, only the 14(15)-DHET was identified following AA-incubation with fasciculata cells, and the (w-1)-hydroxylation was not identified in either incubations. Investigation into the pharmacological activities of the EETs in the fasciculata cells revealed a positive effect on cortisol

production by all four compounds. Maximal stimulation of steriodogenesis was obtained with the 14(15)-isomer at micromolar concentrations.

The synthesis of 5(6)- and 8(9)-EET together with a host of other oxygenated NADPH-dependent AA-metabolites in human isolated epidermal cells has recently been described by Holtzman et al. (1989).

The discovery of a third metabolic pathway for arachidonic acid may have many implications. The EETs have already been shown to have biological properties, the final spectrum of which could be as broad as the eicosanoids. Furthermore, due to the inducible nature of their synthetic pathway, these cytochrome P_{450} products may take a more prominent role where long term administration of P_{450}-inducing drugs or environmental xenobiotics are concerned.

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APPENDIX 1: REAGENTS, BUFFERS AND EQUIPMENT

Reagents:

Acetylcholine chloride - Sigma
Adenosine Diphosphate - Sigma
Arachidonic acid - Sigma
1-^{14}C Arachidonic acid - Amersham
Bovine serum albumin - Sigma
m-Chloroperoxybenzoic acid - Sigma
Indomethacin - Sigma
M&B 22,948 - May & Baker Laboratories (Gift)
Reduced Nicotinamide Adenine Dinucleotide Phosphate (NADPH) - Sigma
b-Naphthoflavone - Sigma
Nordihydroguaiaretic acid (NDGA) - Sigma
Phenobarbitone - Sigma
Phenylephrine HCl - Koch-Light Labs Ltd
Prostacyclin - Welcome (Gift)
Prostaglandin E_1 - Sigma
SKF525A - Smith, Kline & French Laboratories (Gift)
Sodium Nitroprusside - BDH
Thrombin - Sigma

Buffers:

Coomassie G-250 Dye Binding Reagent:
100mg Coomassie G-250 dye dissolved in 50ml of 95% ethanol
add 100ml of 85% w/v phosphoric acid
make to 1l with distilled water.

HEPES-Tyrodes:

NaCl	137mM
KCl	2.7mM
$NaHCO_3$	12mM
NaH_2PO_4	0.4mM
$MgCl_2$	1mM
Glucose	5.5mM
HEPES	10mM

Krebs:

NaCl	118.4mM
Glucose	11.1mM
$NaHCO_3$	25.0mM
$MgSO_4.7H_2O$	1.2mM
KH_2PO_4	1.2mM
$CaCl_2.6H_2O$	2.6mM
KCl	4.7mM

Phosphate Buffered Saline:

11.9g $Na_2HPO_4.2H_2O$ made to 1l with normal saline
9.1g KH_2PO_4 made to 1l with normal saline
final soln -- 85.2ml $Na_2HPO_4.2H_2O$ soln : 14.8ml KH_2PO_4 soln, pH adjusted to 7.4 using HCl or NaOH.

Equipment:

TLC plates - Watman LK5D channelled silica plates, 0.25mm thick, particle size 60 Angstrom.
X-ray Photographic plates - Kodak XAR-351
Developing solutions - Kodak fixer
Kodak Dektol Developer

APPENDIX 2: RESULT TABLES TO ACCOMPANY GRAPHS

Table A3.2: PE-induced contraction of rat aortic strips in the presence and absence of an intact endothelial lining.

PE conc.	Endothelium MEAN ± SEM (n)	No Endothelium MEAN ± SEM (n)
10^{-9}		3.3 ± 1.4 (14)
$3x10^{-9}$	1.7 ± 0.9 (17)	23.8 ± 4.0 (14)
10^{-8}	22.3 ± 2.8 (17)	66.0 ± 1.8 (14)
$3x10^{-8}$	58.3 ± 2.3 (17)	83.8 ± 0.5 (14)
10^{-7}	78.7 ± 1.2 (17)	91.3 ± 0.8 (14)
$3x10^{-7}$	89.7 ± 0.3 (17)	95.8 ± 1.7 (14)
10^{-6}	96.7 ± 0.7 (17)	100.0 ± 0.0 (14)
$3x10^{-6}$	100.0 ± 0.0 (17)	
EC_{50}	$2.5 \pm 0.1 \times 10^{-8}$	$7.3 \pm 0.4 \times 10^{-9}$

Table A3.3: NaNP-induced relaxation of PE pre-contracted rat aortic strips in the presence and absence of an endothelial lining.

NaNP conc.	Endothelium MEAN ± SEM (n)	No Endothelium MEAN ± SEM (n)
10^{-10}	3.7 ± 1.2 (15)	3.3 ± 0.9 (15)
$3x10^{-10}$	12.0 ± 0.6 (15)	10.5 ± 1.8 (15)
10^{-9}	42.3 ± 1.8 (15)	38.8 ± 3.3 (15)
$3x10^{-9}$	72.3 ± 1.8 (15)	70.3 ± 3.1 (15)
10^{-8}	93.3 ± 2.3 (15)	90.5 ± 2.7 (15)
$3x10^{-8}$	101.3 ± 0.9 (15)	97.8 ± 1.6 (15)
10^{-7}	104.0 ± 0.0 (15)	102.3 ± 1.9 (15)
EC_{50}	$1.6 \pm 0.1 \times 10^{-9}$	$1.8 \pm 0.2 \times 10^{-9}$

Table A3.4: EET-induced relaxation of PE pre-contracted rat aortic strips with intact endothelium.

EET conc.	5(6)-EET MEAN ± SEM (n)	8(9)-EET MEAN ± SEM (n)	11(12)-EET MEAN ± SEM (n)	14(15)-EET MEAN ± SEM (n)
$3x10^{-9}$	11.3 ± 3.0 (4)			
10^{-8}	19.0 ± 4.0 (4)			
$3x10^{-8}$	43.3 ± 3.5 (4)			
10^{-7}	88.8 ± 3.1 (4)			
$3x10^{-7}$	98.8 ± 1.6 (4)			
10^{-6}		17.0 ± 2.0 (4)	10.0 ± 2.0 (3)	5.7 ± 1.5 (3)
$3x10^{-6}$		34.3 ± 3.2 (4)	16.3 ± 2.3 (3)	13.3 ± 1.2 (3)
10^{-5}		73.3 ± 5.1 (4)	79.0 ± 1.0 (3)	56.3 ± 0.9 (3)
$3x10^{-5}$		93.0 ± 0.9 (4)	99.3 ± 3.4 (3)	95.3 ± 2.2 (3)
EC_{50}	$3.9 \pm 0.6 \times 10^{-8}$	$5.2 \pm 0.7 \times 10^{-6}$	$6.7 \pm 0.2 \times 10^{-6}$	$8.6 \pm 0.2 \times 10^{-6}$

Table A3.5: EET-induced relaxation of PE pre-contracted rat aortic strips with no endothelial lining.

EET conc.	5(6)-EET MEAN ± SEM (n)	8(9)-EET MEAN ± SEM (n)	11(12)-EET MEAN ± SEM (n)	14(15)-EET MEAN ± SEM (n)
10^{-8}	12.0 ± 3.9 (4)			
$3x10^{-8}$	27.3 ± 7.2 (4)			
10^{-7}	56.0 ± 7.6 (4)			
$3x10^{-7}$	84.3 ± 2.1 (4)			
10^{-6}	97.0 ± 2.5 (4)	16.0 ± 9.5 (3)	5.0 ± 2.1 (3)	1.7 ± 1.7 (3)
$3x10^{-6}$		36.0 ± 9.0 (3)	18.0 ± 2.0 (3)	7.7 ± 4.4 (3)
10^{-5}		73.0 ± 8.0 (3)	80.0 ± 1.5 (3)	56.7 ± 6.4 (3)
$3x10^{-5}$		90.3 ± 5.2 (3)	98.0 ± 2.1 (3)	98.0 ± 4.0 (3)
EC_{50}	$8.2 \pm 1.8 \times 10^{-8}$	$4.7 \pm 1.7 \times 10^{-6}$	$6.5 \pm 0.2 \times 10^{-6}$	$8.9 \pm 0.8 \times 10^{-6}$

Table A3.7a: EET-induced relaxation of PE pre-contracted intact rat aortic strips in the presence of $3x10^{-6}$M indomethacin.

EET conc.	5(6)-EET MEAN ± SEM (n)	8(9)-EET MEAN ± SEM (n)	11(12)-EET MEAN ± SEM (n)	14(15)-EET MEAN ± SEM (n)
10^{-8}	4.0 ± 2.6 (4)			
$3x10^{-8}$	23.3 ± 3.4 (4)			
10^{-7}	59.5 ± 6.2 (4)			
$3x10^{-7}$	80.3 ± 3.6 (4)			
10^{-6}	92.8 ± 4.2 (4)	14.0 ± 3.0 (4)	9.0 ± 3.5 (4)	6.5 ± 3.8 (4)
$3x10^{-6}$		41.0 ± 7.3 (4)	20.8 ± 4.7 (4)	14.0 ± 3.5 (4)
10^{-5}		71.5 ± 6.4 (4)	74.5 ± 6.2 (4)	58.0 ± 5.8 (4)
$3x10^{-5}$		95.5 ± 4.2 (4)	97.8 ± 1.3 (4)	97.0 ± 3.0 (4)
EC_{50}	$7.5 \pm 1.0 \times 10^{-8}$	$4.5 \pm 1.6 \times 10^{-6}$	$6.7 \pm 0.7 \times 10^{-6}$	$8.5 \pm 0.8 \times 10^{-6}$

Table A3.7b: EET-induced relaxation of PE pre-contracted de-endothelialised rat aortic strips in the presence of $3x10^{-6}$M indomethacin.

EET conc.	5(6)-EET MEAN ± SEM (n)	8(9)-EET MEAN ± SEM (n)	11(12)-EET MEAN ± SEM (n)	14(15)-EET MEAN ± SEM (n)
10^{-7}	11.3 ± 3.9 (4)			
$3x10^{-7}$	24.5 ± 6.0 (4)			
10^{-6}	42.5 ± 6.8 (4)	19.3 ± 7.2 (3)	5.3 ± 5.3 (3)	5.0 ± 2.6 (3)
$3x10^{-6}$	86.0 ± 5.2 (4)	29.3 ± 5.8 (3)	17.0 ± 4.0 (3)	10.7 ± 5.3 (3)
10^{-5}	92.8 ± 4.6 (4)	78.0 ± 6.7 (3)	77.0 ± 6.1 (3)	62.3 ± 9.3 (3)
$3x10^{-5}$		87.3 ± 4.7 (3)	93.0 ± 3.6 (3)	96.3 ± 3.7 (3)
EC_{50}	$1.2 \pm 0.3 \times 10^{-6}$	$5.1 \pm 0.9 \times 10^{-6}$	$6.4 \pm 0.5 \times 10^{-6}$	$8.1 \pm 1.1 \times 10^{-6}$

Table A3.8: EET-induced relaxation of intact PE pre-contracted rat aortic strips exposed to $1x10^{-6}$M NDGA.

EET conc.	5(6)-EET MEAN ± SEM (n)	8(9)-EET MEAN ± SEM (n)	11(12)-EET MEAN ± SEM (n)	14(15)-EET MEAN ± SEM (n)
$3x10^{-9}$	12.8 ± 3.3 (4)			
10^{-8}	20.8 ± 4.2 (4)			
$3x10^{-8}$	47.8 ± 3.5 (4)			
10^{-7}	87.5 ± 3.3 (4)			
$3x10^{-7}$	96.3 ± 2.1 (4)			
10^{-6}		15.0 ± 3.6 (4)	15.5 ± 2.9 (4)	14.8 ± 3.2 (4)
$3x10^{-6}$		35.3 ± 4.5 (4)	25.5 ± 4.9 (4)	23.0 ± 4.8 (4)
10^{-5}		73.0 ± 4.5 (4)	78.3 ± 4.0 (4)	56.3 ± 5.5 (4)
$3x10^{-5}$		90.3 ± 3.0 (4)	94.3 ± 1.9 (4)	90.8 ± 2.3 (4)
EC_{50}	$3.1 \pm 0.7 \times 10^{-8}$	$4.8 \pm 0.8 \times 10^{-6}$	$5.9 \pm 0.6 \times 10^{-6}$	$7.7 \pm 1.1 \times 10^{-6}$

Table A3.9: PE-induced contraction of rat aortic strips with an intact endothelial lining in the presence and absence of $3x10^{-6}$M M&B 22,948.

PE conc.	No M&B 22,948 MEAN ± SEM (n)	$3x10^{-6}$ M&B 22,948 MEAN ± SEM (n)
10^{-9}	0.8 ± 0.5 (6)	
$3x10^{-9}$	5.0 ± 1.1 (6)	
10^{-8}	50.8 ± 5.4 (6)	
$3x10^{-8}$	74.8 ± 3.1 (6)	4.5 ± 1.3 (6)
10^{-7}	89.0 ± 2.0 (6)	35.3 ± 2.5 (6)
$3x10^{-7}$	95.3 ± 1.1 (6)	73.8 ± 3.9 (6)
10^{-6}	99.5 ± 0.5 (6)	90.0 ± 4.4 (6)
$3x10^{-6}$		98.8 ± 1.3 (6)
EC_{50}	$9.8 \pm 0.7 \times 10^{-9}$	$1.7 \pm 0.1 \times 10^{-7}$

Table A3.10: ACh-induced relaxation of PE pre-contracted rat aortic strips with an intact endothelial lining in the presence and absence of $3x10^{-6}$M M&B 22,948.

ACh conc.	No M&B 22,948 MEAN ± SEM (n)	$3x10^{-6}$ M&B 22,948 MEAN ± SEM (n)
10^{-10}		3.8 ± 1.9 (6)
$3x10^{-10}$		11.0 ± 2.6 (6)
10^{-9}	4.8 ± 1.5 (6)	18.3 ± 4.1 (6)
$3x10^{-9}$	10.8 ± 2.5 (6)	48.5 ± 3.5 (6)
10^{-8}	39.0 ± 4.0 (6)	82.5 ± 4.1 (6)
$3x10^{-8}$	70.3 ± 3.5 (6)	93.8 ± 2.2 (6)
10^{-7}	91.0 ± 1.5 (6)	98.3 ± 1.2 (6)
$3x10^{-7}$	97.0 ± 1.2 (6)	
10^{-6}	99.8 ± 0.3 (6)	
EC_{50}	$1.7 \pm 0.3 \times 10^{-8}$	$3.1 \pm 0.7 \times 10^{-9}$

Table A3.11: NaNP-induced relaxation of PE pre-contracted rat aortic strips with an intact endothelial lining in the presence and absence of $3x10^{-6}$M M&B 22,948.

NaNP conc.	No M&B 22,948 MEAN ± SEM (n)	$3x10^{-6}$ M&B 22,948 MEAN ± SEM (n)
10^{-11}		1.3 ± 0.8 (6)
$3x10^{-11}$		6.0 ± 1.8 (6)
10^{-10}	2.0 ± 0.8 (6)	14.0 ± 2.1 (6)
$3x10^{-10}$	13.3 ± 1.3 (6)	31.8 ± 3.6 (6)
10^{-9}	32.5 ± 3.8 (6)	64.0 ± 3.8 (6)
$3x10^{-9}$	70.8 ± 3.1 (6)	88.3 ± 2.9 (6)
10^{-8}	94.0 ± 2.1 (6)	97.0 ± 1.7 (6)
$3x10^{-8}$	99.8 ± 1.5 (6)	100.8 ± 2.3 (6)
10^{-7}	102.3 ± 1.0 (6)	
EC_{50}	$2.0 \pm 0.2 \times 10^{-9}$	$7.0 \pm 0.8 \times 10^{-10}$

Table A3.12: EET-induced relaxation of de-endothelialised rat thoracic aortic strips in the absence of M&B22,948.

EET conc.	5(6)-EET MEAN ± SEM (n)	8(9)-EET MEAN ± SEM (n)	11(12)-EET MEAN ± SEM (n)	14(15)-EET MEAN ± SEM (n)
$3x10^{-9}$	2.3 ± 1.7 (4)			
10^{-8}	15.0 ± 3.7 (4)			
$3x10^{-8}$	32.8 ± 5.9 (4)			
10^{-7}	54.5 ± 4.3 (4)			
$3x10^{-7}$	87.3 ± 3.9 (4)	4.3 ± 3.3 (4)		1.3 ± 0.8 (4)
10^{-6}	95.8 ± 2.7 (4)	12.8 ± 4.6 (4)	4.3 ± 2.6 (4)	9.3 ± 3.5 (4)
$3x10^{-6}$		35.8 ± 4.0 (4)	25.8 ± 4.2 (4)	27.3 ± 5.0 (4)
10^{-5}		69.5 ± 6.0 (4)	74.3 ± 5.9 (4)	64.8 ± 6.0 (4)
$3x10^{-5}$		89.8 ± 4.1 (4)	94.3 ± 2.8 (4)	92.8 ± 3.8 (4)
EC_{50}	$8.5 \pm 1.6 \times 10^{-8}$	$5.9 \pm 0.9 \times 10^{-6}$	$6.5 \pm 0.7 \times 10^{-6}$	$7.2 \pm 1.0 \times 10^{-6}$

Table A3.12: EET-induced relaxation of de-endothelialised rat thoracic aortic strips in the presence of M&B22,948.

EET conc.	5(6)-EET MEAN ± SEM (n)	8(9)-EET MEAN ± SEM (n)	11(12)-EET MEAN ± SEM (n)	14(15)-EET MEAN ± SEM (n)
$3x10^{-9}$	6.3 ± 2.4 (4)			
10^{-8}	19.0 ± 4.4 (4)			
$3x10^{-8}$	32.0 ± 5.9 (4)			
10^{-7}	56.3 ± 6.2 (4)	11.5 ± 3.7 (4)	3.5 ± 2.3 (4)	6.5 ± 4.5 (4)
$3x10^{-7}$	82.8 ± 5.1 (4)	25.5 ± 5.0 (4)	13.3 ± 2.6 (4)	19.0 ± 3.4 (4)
10^{-6}	97.0 ± 2.1 (4)	40.8 ± 6.7 (4)	23.5 ± 4.7 (4)	32.8 ± 5.7 (4)
$3x10^{-6}$		86.5 ± 4.2 (4)	80.5 ± 4.7 (4)	73.8 ± 4.6 (4)
10^{-5}		92.3 ± 2.8 (4)	93.3 ± 2.5 (4)	91.8 ± 3.9 (4)
EC_{50}	$7.8 \pm 1.8 \times 10^{-8}$	$1.2 \pm 0.3 \times 10^{-6}$	$1.8 \pm 0.2 \times 10^{-6}$	$1.6 \pm 0.2 \times 10^{-6}$

Table A3.14: Acetylcholine-induced contraction of guinea-pig tracheal rings in the presence and absence of $3x10^{-6}$M Indomethacin.

ACh conc.	No Indomethacin MEAN ± SEM (n)	$3x10^{-6}$ Indo. MEAN ± SEM (n)
10^{-7}	0.22 ± 0.04 (11)	0.15 ± 0.05 (13)
$3x10^{-7}$	0.50 ± 0.07 (11)	0.52 ± 0.11 (13)
10^{-6}	0.92 ± 0.13 (11)	1.22 ± 0.18 (13)
$3x10^{-6}$	1.37 ± 0.20 (11)	2.02 ± 0.23 (13)
10^{-5}	1.83 ± 0.25 (11)	2.77 ± 0.27 (13)
$3x10^{-5}$	2.33 ± 0.29 (11)	3.42 ± 0.30 (13)
10^{-4}	2.88 ± 0.39 (11)	3.73 ± 0.38 (13)

Table A3.15: NaNP-induced relaxation of guinea-pig tracheal rings in the presence and absence of $3x10^{-6}$M Indomethacin.

NaNP conc.	No Indomethacin MEAN ± SEM (n)	$3x10^{-6}$ Indo. MEAN ± SEM (n)
10^{-7}	5.1 ± 0.8 (7)	4.6 ± 1.1 (8)
10^{-6}	44.9 ± 2.7 (7)	29.1 ± 2.8 (8)
10^{-5}	93.3 ± 4.4 (7)	68.2 ± 2.4 (8)
10^{-4}	99.7 ± 5.1 (7)	74.8 ± 2.2 (8)
10^{-3}	100.4 ± 4.3 (7)	75.6 ± 2.4 (8)
EC_{50}	$2.0 \pm 0.5 \times 10^{-6}$	$3.0 \pm 0.6 \times 10^{-6}$

Table A3.16: EET-induced relaxation of ACh pre-contracted guinea-pig tracheal rings in the absence of $3x10^{-6}$M Indomethacin.

EET conc.	5(6)-EET MEAN ± SEM (n)	8(9)-EET MEAN ± SEM (n)	11(12)-EET MEAN ± SEM (n)	14(15)-EET MEAN ± SEM (n)
10^{-7}	3.0 ± 1.2 (8)			
$3x10^{-7}$	8.0 ± 3.6 (8)			
10^{-6}	27.4 ± 4.7 (8)			
$3x10^{-6}$	44.8 ± 3.7 (8)		13.8 ± 8.5 (5)	
10^{-5}	55.6 ± 5.3 (8)	2.0 ± 0.3 (5)	22.8 ± 9.3 (5)	5.6 ± 1.5 (4)
$3x10^{-5}$	60.6 ± 5.0 (8)	9.6 ± 4.3 (5)	30.8 ± 7.5 (5)	14.3 ± 1.7 (4)
10^{-4}		42.6 ± 0.7 (5)	48.0 ± 11.0 (5)	46.3 ± 1.1 (4)
$3x10^{-4}$		93.0 ± 12.1 (5)	98.6 ± 0.6 (5)	72.5 ± 6.5 (4)
10^{-3}				82.5 ± 5.5 (4)
EC_{50}	$1.3 \pm 0.5 \times 10^{-6}$	$7.3 \pm 0.4 \times 10^{-5}$	$2.8 \pm 1.6 \times 10^{-5}$	$6.4 \pm 0.3 \times 10^{-5}$

Table A3.17: EET-induced relaxation of ACh pre-contracted guinea-pig tracheal rings in the presence of $3x10^{-6}$M Indomethacin.

EET conc.	5(6)-EET MEAN ± SEM (n)	8(9)-EET MEAN ± SEM (n)	11(12)-EET MEAN ± SEM (n)	14(15)-EET MEAN ± SEM (n)
10^{-6}	5.3 ± 4.0 (4)			
$3x10^{-6}$	11.3 ± 4.9 (4)			
10^{-5}	28.0 ± 11.9 (4)	7.0 ± 4.4 (3)	4.0 ± 1.0 (3)	4.0 ± 3.1 (3)
$3x10^{-5}$	32.3 ± 2.3 (4)	7.0 ± 4.4 (3)	16.3 ± 5.2 (3)	11.0 ± 2.1 (3)
10^{-4}	63.0 ± 3.3 (4)	33.8 ± 6.0 (3)	40.0 ± 10.5 (3)	35.0 ± 7.8 (3)
$3x10^{-4}$	76.3 ± 4.0 (4)	88.0 ± 13.9 (3)	89.3 ± 10.7 (3)	54.5 ± 13.5 (3)
10^{-3}				69.5 ± 11.5 (3)
EC_{50}	$4.3 \pm 0.6 \times 10^{-5}$	$1.4 \pm 0.3 \times 10^{-4}$	$1.2 \pm 0.3 \times 10^{-4}$	$9.9 \pm 1.8 \times 10^{-5}$

Table A3.20: EET-induced relaxation of ACh-precontracted guinea-pig tracheal rings in the presence of $1x10^{-6}$M NDGA.

EET conc.	5(6)-EET MEAN ± SEM (n)	8(9)-EET MEAN ± SEM (n)	11(12)-EET MEAN ± SEM (n)	14(15)-EET MEAN ± SEM (n)
10^{-7}	6.8 ± 3.2 (4)			
$3x10^{-7}$	9.3 ± 1.9 (4)			
10^{-6}	28.8 ± 3.4 (4)			
$3x10^{-6}$	43.8 ± 6.1 (4)			
10^{-5}	51.8 ± 5.8 (4)	3.5 ± 2.9 (4)	14.3 ± 2.9 (4)	3.8 ± 2.3 (4)
$3x10^{-5}$	56.0 ± 3.6 (4)	11.5 ± 4.3 (4)	25.5 ± 5.5 (4)	18.3 ± 5.1 (4)
10^{-4}		48.0 ± 5.1 (4)	50.8 ± 5.6 (4)	43.8 ± 5.4 (4)
$3x10^{-4}$		89.5 ± 4.3 (4)	96.8 ± 3.0 (4)	77.0 ± 6.3 (4)
10^{-3}		97.3 ± 2.2 (4)	97.8 ± 2.3 (4)	84.0 ± 5.0 (4)
EC_{50}	$9.7 \pm 1.1 \times 10^{-7}$	$1.0 \pm 0.1 \times 10^{-4}$	$9.5 \pm 1.6 \times 10^{-5}$	$9.5 \pm 1.5 \times 10^{-5}$

Table A3.21: NaNP-induced relaxation of guinea-pig tracheal rings in the presence and absence of $1x10^{-6}$M M&B 22,948

NaNP conc.	No M&B 22,948 MEAN ± SEM (n)	$1x10^{-6}$M M&B22,948 MEAN ± SEM (n)
10^{-9}		3.0 ± 2.7 (4)
10^{-8}	3.8 ± 1.8 (4)	7.8 ± 1.7 (4)
10^{-7}	6.5 ± 2.5 (4)	21.3 ± 6.4 (4)
10^{-6}	47.5 ± 6.5 (4)	87.0 ± 4.8 (4)
10^{-5}	91.5 ± 3.3 (4)	96.0 ± 2.3 (4)
10^{-4}	98.0 ± 2.0 (4)	
EC_{50}	$1.3 \pm 0.4 \times 10^{-6}$	$4.7 \pm 0.8 \times 10^{-7}$

Table A3.22: EET-induced relaxation of ACh-precontracted guinea-pig tracheal rings in the absence of M&B 22,948.

EET conc.	5(6)-EET MEAN ± SEM (n)	8(9)-EET MEAN ± SEM (n)	11(12)-EET MEAN ± SEM (n)	14(15)-EET MEAN ± SEM (n)
10^{-7}	4.5 ± 2.1 (4)			
$3x10^{-7}$	10.8 ± 4.1 (4)			
10^{-6}	26.3 ± 4.2 (4)			
$3x10^{-6}$	41.5 ± 4.5 (4)			
10^{-5}	52.5 ± 3.3 (4)	8.0 ± 3.6 (4)	12.8 ± 3.1 (4)	3.0 ± 1.9 (4)
$3x10^{-5}$	58.8 ± 2.7 (4)	10.8 ± 4.0 (4)	27.3 ± 4.7 (4)	10.8 ± 6.1 (4)
10^{-4}		41.0 ± 4.6 (4)	44.0 ± 6.0 (4)	47.3 ± 4.5 (4)
$3x10^{-4}$		92.5 ± 5.0 (4)	90.0 ± 4.6 (4)	78.8 ± 4.9 (4)
10^{-3}		96.5 ± 2.9 (4)	96.3 ± 2.2 (4)	86.3 ± 3.8 (4)
EC_{50}	$1.4 \pm 0.6 \times 10^{-6}$	$1.3 \pm 0.2 \times 10^{-4}$	$1.2 \pm 0.3 \times 10^{-4}$	$9.2 \pm 0.9 \times 10^{-5}$

Table A3.22: EET-induced relaxation of ACh-precontracted guinea-pig tracheal rings in the presence of $1x10^{-6}$M M&B 22,948.

EET conc.	5(6)-EET MEAN ± SEM (n)	8(9)-EET MEAN ± SEM (n)	11(12)-EET MEAN ± SEM (n)	14(15)-EET MEAN ± SEM (n)
10^{-7}	4.3 ± 1.9 (4)			
$3x10^{-7}$	7.0 ± 3.4 (4)			
10^{-6}	20.5 ± 4.0 (4)	5.0 ± 2.4 (4)	12.3 ± 1.8 (4)	6.3 ± 2.8 (4)
$3x10^{-6}$	39.3 ± 4.2 (4)	13.8 ± 4.8 (4)	19.3 ± 4.8 (4)	11.8 ± 4.6 (4)
10^{-5}	55.3 ± 4.2 (4)	24.5 ± 4.7 (4)	33.3 ± 5.0 (4)	23.3 ± 4.1 (4)
$3x10^{-5}$	60.5 ± 2.5 (4)	34.0 ± 4.6 (4)	46.0 ± 4.9 (4)	37.8 ± 6.5 (4)
10^{-4}		82.5 ± 5.5 (4)	89.5 ± 5.5 (4)	76.8 ± 2.9 (4)
$3x10^{-4}$		94.0 ± 3.5 (4)	97.8 ± 2.3 (4)	83.8 ± 2.6 (4)
EC_{50}	$2.0 \pm 0.4 \times 10^{-6}$	$4.9 \pm 0.7 \times 10^{-5}$	$3.5 \pm 0.8 \times 10^{-5}$	$3.7 \pm 1.2 \times 10^{-5}$

Table A4.1: ADP-induced aggregation of rat platelet rich plasma

ADP conc.	% Aggregation MEAN ± SEM (n)
4×10^{-6}	1.5 ± 1.2 (7)
8×10^{-6}	24.8 ± 4.1 (7)
1.2×10^{-5}	45.0 ± 3.0 (7)
1.6×10^{-5}	64.5 ± 3.7 (7)
2×10^{-5}	85.5 ± 5.0 (7)
3×10^{-5}	98.0 ± 2.0 (7)
EC_{50}	$1.3 \pm 0.1 \times 10^{-5}$

Table A4.2: EET inhibition of rat platelet rich plasma aggregation induced by ADP

EET conc.	5(6)-EET MEAN ± SEM (n)	8(9)-EET MEAN ± SEM (n)	11(12)-EET MEAN ± SEM (n)	14(15)-EET MEAN ± SEM (n)
10^{-6}	2.5 ± 2.5 (4)			
10^{-5}	28.3 ± 4.8 (4)		10.5 ± 1.8 (4)	9.5 ± 4.6 (4)
1.5×10^{-5}	53.3 ± 5.3 (4)			
2×10^{-5}	78.8 ± 4.1 (4)			
3×10^{-5}	98.0 ± 1.1 (4)	7.0 ± 2.0 (4)	23.0 ± 4.9 (4)	26.3 ± 3.9 (4)
5×10^{-5}		19.0 ± 4.7 (4)	36.5 ± 4.8 (4)	42.8 ± 4.4 (4)
7×10^{-5}		34.5 ± 5.9 (4)	75.3 ± 5.4 (4)	60.8 ± 5.3 (4)
10^{-4}		61.3 ± 2.5 (4)	93.0 ± 3.3 (4)	92.5 ± 2.9 (4)
2×10^{-4}		90.0 ± 4.2 (4)	98.8 ± 1.3 (4)	98.5 ± 1.2 (4)
EC_{50}	$1.4 \pm 0.1 \times 10^{-5}$	$8.2 \pm 0.6 \times 10^{-5}$	$5.7 \pm 0.3 \times 10^{-5}$	$5.7 \pm 0.5 \times 10^{-5}$

Table A4.3: ADP-induced aggregation of rat washed platelets

ADP conc.	% Aggregation MEAN ± SEM (n)
3×10^{-7}	0.8 ± 0.5 (10)
4×10^{-7}	17.8 ± 4.2 (10)
5×10^{-7}	56.8 ± 3.6 (10)
7×10^{-7}	74.0 ± 2.5 (10)
10^{-6}	95.0 ± 3.2 (10)
3×10^{-6}	99.8 ± 0.3 (10)
EC_{50}	$4.8 \pm 0.1 \times 10^{-7}$

Table A4.4: Thrombin-induced aggregation of rat washed platelets (conc. in NIH units/ml).

Thromb. conc.	% Aggregation MEAN ± SEM (n)
0.053	0.5 ± 0.5 (8)
0.106	12.3 ± 3.1 (8)
0.212	28.3 ± 2.7 (8)
0.424	61.8 ± 3.4 (8)
0.636	96.5 ± 2.4 (8)
0.848	100.0 ± 0.0 (8)
EC_{50}	0.349 ± 0.019

Table A4.5: EET inhibition of rat washed-platelet aggregation induced by ADP

EET conc.	5(6)-EET MEAN ± SEM (n)	8(9)-EET MEAN ± SEM (n)	11(12)-EET MEAN ± SEM (n)	14(15)-EET MEAN ± SEM (n)
10^{-7}	25.5 ± 7.4 (4)			
$5x10^{-7}$	52.8 ± 8.0 (4)			
10^{-6}	84.0 ± 6.7 (4)	9.7 ± 9.7 (3)	7.0 ± 7.0 (3)	5.0 ± 2.5 (3)
$1.5x10^{-6}$	92.8 ± 3.8 (4)			
$2x10^{-6}$	95.8 ± 2.4 (4)			
$5x10^{-6}$		33.3 ± 12.5 (3)	35.3 ± 13.7 (3)	35.3 ± 8.7 (3)
10^{-5}		66.7 ± 8.1 (3)	68.0 ± 8.4 (3)	70.0 ± 12.5 (3)
$1.5x10^{-5}$		81.7 ± 5.2 (3)	86.7 ± 6.3 (3)	76.7 ± 0.9 (3)
$2x10^{-5}$		88.0 ± 6.0 (3)	93.0 ± 4.0 (3)	81.0 ± 3.0 (3)
EC_{50}	$4.2 \pm 1.2 \times 10^{-7}$	$6.6 \pm 1.9 \times 10^{-6}$	$6.7 \pm 2.0 \times 10^{-6}$	$5.7 \pm 1.1 \times 10^{-6}$

Table A4.6: EET inhibition of rat washed-platelet aggregation induced by Thrombin.

EET conc.	5(6)-EET MEAN ± SEM (n)	8(9)-EET MEAN ± SEM (n)	11(12)-EET MEAN ± SEM (n)	14(15)-EET MEAN ± SEM (n)
10^{-7}	1.3 ± 0.9 (4)			
$5x10^{-7}$	39.8 ± 13.1 (4)			
10^{-6}	69.5 ± 10.5 (4)			
$1.5x10^{-6}$	81.8 ± 6.4 (4)			
$2x10^{-6}$	100.0 ± 0.0 (4)			
$5x10^{-6}$				3.7 ± 3.7 (3)
10^{-5}		2.3 ± 1.9 (3)	1.0 ± 1.0 (3)	28.7 ± 17.4 (3)
$2x10^{-5}$		9.3 ± 2.3 (3)	37.7 ± 7.5 (3)	63.3 ± 13.6 (3)
$3x10^{-5}$		37.0 ± 8.1 (3)	86.3 ± 8.2 (3)	87.0 ± 7.2 (3)
$4x10^{-5}$		47.0 ± 5.8 (3)	95.0 ± 1.0 (3)	98.0 ± 2.0 (3)
$5x10^{-5}$		94.0 ± 5.0 (3)	97.6 ± 1.9 (3)	100.0 ± 0.0 (3)
$6x10^{-5}$		96.9 ± 1.0 (3)		
EC_{50}	$6.7 \pm 1.9 \times 10^{-7}$	$4.0 \pm 0.8 \times 10^{-5}$	$2.2 \pm 0.3 \times 10^{-5}$	$1.6 \pm 0.5 \times 10^{-5}$

Table A4.8: The effect of Indomethacin on EET-induced anti-aggregatory activity.

EET	5(6)-EET	8(9)-EET	11(12)-EET	14(15)-EET
	MEAN ± SEM (n)	MEAN ± SEM (n)	MEAN ± SEM (n)	MEAN ± SEM (n)
change	15.8 ± 11.0 (3)	11.3 ± 4.2 (3)	7.1 ± 3.5 (3)	18.9 ± 2.4 (3)

Table A4.9: The effect of NDGA on EET-induced anti-aggregatory activity.

EET	5(6)-EET	8(9)-EET	11(12)-EET	14(15)-EET
	MEAN ± SEM (n)	MEAN ± SEM (n)	MEAN ± SEM (n)	MEAN ± SEM (n)
change	43.9 ± 6.2 (3)	17.1 ± 1.1 (3)	22.9 ± 2.9 (3)	27.6 ± 1.8 (3)

Table A4.11: PGE_1-induced inhibition of rat washed-platelet aggregation induced by ADP

PGE_1 conc.	MEAN ± SEM (n)
10^{-9}	9.5 ± 3.4 (4)
$3x10^{-9}$	22.3 ± 6.0 (4)
10^{-8}	49.5 ± 6.4 (4)
$3x10^{-8}$	73.5 ± 5.0 (4)
10^{-7}	83.5 ± 5.7 (4)
$3x10^{-7}$	93.0 ± 3.4 (4)
EC_{50}	$1.0 \pm 0.5 \times 10^{-8}$

Table A4.12: PGI_2-induced inhibition of rat washed-platelet aggregation induced by ADP

PGI_2 conc.	MEAN ± SEM (n)
$3x10^{-10}$	5.8 ± 0.9 (6)
10^{-9}	11.5 ± 2.1 (6)
$3x10^{-9}$	45.8 ± 3.4 (6)
$6x10^{-9}$	72.5 ± 6.0 (6)
10^{-8}	94.3 ± 1.4 (6)
$3x10^{-8}$	99.8 ± 0.3 (6)
EC_{50}	$3.5 \pm 0.4 \times 10^{-9}$

Table A4.14: The effect of Papaverine on PGI_2-induced anti-aggregatory activity.

EET	PGI^2
	MEAN ± SEM (n)
change	32.8 ± 0.9 (4)

Table A4.13: NaNP-induced inhibition of rat washed-platelet aggregation induced by ADP

NaNP conc.	MEAN ± SEM (n)
$3x10^{-6}$	2.0 ± 0.7 (8)
10^{-5}	24.0 ± 3.9 (8)
$3x10^{-5}$	43.3 ± 3.9 (8)
10^{-4}	74.3 ± 3.1 (8)
$3x10^{-4}$	88.5 ± 2.9 (8)
10^{-3}	95.0 ± 1.1 (8)
EC_{50}	$3.9 \pm 0.8 \times 10^{-5}$

Table A4.15: The effect of M&B 22,948 on NaNP-induced anti-aggregatory activity.

EET	NaNP MEAN ± SEM (n)
change	43.0 ± 2.1 (4)

Table A4.14: The effect of Papaverine on EET-induced anti-aggregatory activity.

EET	5(6)-EET MEAN ± SEM (n)	8(9)-EET MEAN ± SEM (n)	11(12)-EET MEAN ± SEM (n)	14(15)-EET MEAN ± SEM (n)
change	31.5 ± 1.3 (4)	17.0 ± 1.1 (4)	43.3 ± 1.7 (4)	24.3 ± 0.6 (4)

Table A4.15: The effect of M&B 22,948 on EET-induced anti-aggregatory activity.

EET	5(6)-EET MEAN ± SEM (n)	8(9)-EET MEAN ± SEM (n)	11(12)-EET MEAN ± SEM (n)	14(15)-EET MEAN ± SEM (n)
change	1.3 ± 1.0 (4)	0.5 ± 1.6 (4)	1.3 ± 1.2 (4)	2.0 ± 1.0 (4)

Table A4.16: Half-response time of PGI_2 in platelet buffer solution determined by platelet aggregation.

Time (secs)	MEAN ± SEM (n)
0	100.0 ± 0.0 (4)
15	86.0 ± 2.1 (4)
30	68.0 ± 1.4 (4)
60	43.5 ± 4.3 (4)
90	21.5 ± 2.0 (4)
120	4.8 ± 1.6 (4)
180	0.0 ± 0.0 (4)
	52.0 ± 4.9 secs

Table A4.17: Half-response time of the EETs in platelet buffer solution determined by platelet aggregation.

TIME (mins)	5(6)-EET MEAN ± SEM (n)	8(9)-EET MEAN ± SEM (n)	11(12)-EET MEAN ± SEM (n)	14(15)-EET MEAN ± SEM (n)
1	87.0 ± 2.1 (4)	91.5 ± 1.6 (4)	90.0 ± 2.6 (4)	95.5 ± 2.4 (4)
2	78.5 ± 3.0 (4)	81.5 ± 2.0 (4)	81.8 ± 2.7 (4)	89.3 ± 2.2 (4)
3	63.0 ± 4.4 (4)	68.5 ± 3.7 (4)	70.0 ± 3.7 (4)	79.5 ± 4.2 (4)
5	53.5 ± 4.4 (4)	54.0 ± 3.8 (4)	54.0 ± 4.3 (4)	59.5 ± 3.6 (4)
10	39.5 ± 2.9 (4)	41.3 ± 2.5 (4)	36.8 ± 1.5 (4)	44.0 ± 3.5 (4)
15	38.0 ± 3.3 (4)	32.5 ± 1.3 (4)	29.5 ± 2.0 (4)	33.3 ± 3.2 (4)
20	39.8 ± 2.1 (4)	29.8 ± 2.1 (4)	27.8 ± 0.9 (4)	29.8 ± 2.3 (4)
25				28.5 ± 1.0 (4)
	6.3 ± 1.3 mins	6.6 ± 1.2 mins	6.2 ± 0.9 mins	8.1 ± 1.1 mins

Table A4.18: Adjusted half-response time of the EETs in platelet buffer solution determined by platelet aggregation.

TIME (mins)	5(6)-EET MEAN ± SEM (n)	8(9)-EET MEAN ± SEM (n)	11(12)-EET MEAN ± SEM (n)	14(15)-EET MEAN ± SEM (n)
1	79.0 ± 3.4 (4)	87.9 ± 2.3 (4)	86.1 ± 3.6 (4)	93.7 ± 3.4 (4)
2	65.3 ± 3.8 (4)	73.6 ± 2.8 (4)	74.8 ± 3.7 (4)	85.0 ± 2.9 (4)
3	40.3 ± 7.1 (4)	55.1 ± 5.3 (4)	58.4 ± 5.1 (4)	71.3 ± 5.9 (4)
5	25.0 ± 7.1 (4)	34.5 ± 5.4 (4)	36.3 ± 6.0 (4)	43.4 ± 5.0 (4)
10	2.4 ± 4.7 (4)	16.4 ± 3.6 (4)	12.5 ± 2.1 (4)	21.7 ± 4.9 (4)
15	0.0 ± 5.3 (4)	3.8 ± 1.9 (4)	2.4 ± 2.8 (4)	6.7 ± 4.5 (4)
20		0.0 ± 3.0 (4)	0.0 ± 1.2 (4)	1.8 ± 3.2 (4)
25				0.0 ± 1.4 (4)
	2.6 ± 0.3 mins	3.5 ± 0.5 mins	3.8 ± 0.5 mins	4.5 ± 0.4 mins

Table A4.18: 'Half-life' of the EETs determined using inhibition of platelet aggregation.

	5(6)-EET	8(9)-EET	11(12)-EET	14(15)-EET
70% Resp. Time1 (Range) secs.	99 (84-116)	132 (120-144)	138 (124-154)	186 (160-210)
70% Resp. Conc.	7.8×10^{-7}	1.1×10^{-5}	1.1×10^{-5}	1.0×10^{-5}
Half-conc.	3.9×10^{-7}	5.5×10^{-6}	5.5×10^{-6}	5.0×10^{-6}
Half-conc. Resp.	45	37	39	35
Half-conc. Time2 (Range) secs.	169 (155-187)	285 (254-317)	285 (255-319)	416 (347-484)
Time 1 - Time 2 (Range) secs.	70 (39-103)	153 (110-197)	147 (101-195)	230 (137-324)

Table A4.16: 'Half-life' of PGI_2 determined using inhibition of platelet aggregation.

	PGI2
70% Resp. Time1 (Range) secs.	28 (27-29)
70% Resp. Conc.	5.7×10^{-9}
Half-conc.	2.9×10^{-9}
Half-conc. Resp.	44
Half-conc. Time2 (Range) secs.	59 (55-65)
Time 1 - Time 2 (Range) secs.	31 (26-38)

APPENDIX 3: PUBLICATIONS

CLEGG M.F., FINNEN M.J. & WILLIAMS K.I. (1989) Biological Activities of the Epoxides of Arachidonic Acid. Br. J. Pharmacol. 98, 906P.